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The Parental effect in the immune system

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Abbreviations

TCR – T cell receptor
Ab – Antibodies
IL – Interleukin
CD – Cluster of Differentiation
OVA – Ovalbumin
HDM – house dust mite
N.b. – *Nippostrongylus brasiliensis*
ABX – Antibiotic
p.i. – Post infection
DCs – Dendritic Cells
APC – Antigen-presenting cell
ILC – Innate Lymphoid Cells
MC – Mast Cell
Ig – Immunoglobulin
LMC – Littermate control
KO – Knockout
s.c. – Subcutaneous
WT – wild type
c-sec – C-section/cesarean
BAL – bronchoalveolar lavage
FcRn – neonatal Fc receptor
AM – alveolar macrophage
Treg – regulatory T cell
Th – T helper cell
CTL- Cytotoxic T lymphocyte
MLN – Mesenteric Lymph Nodes
SCFA – short chain fatty acids
GF – germ-free
SPF – specific pathogen-free
Tfh – T follicular helper cell
ADCC – Antibody-dependent cellular cytotoxicity
M2 – Alternatively activated macrophages

Resumo

Durante a gestação e a amamentação, diversos fatores maternos podem influenciar o sistema imunitário dos filhos, levando a diversas alterações que terão um impacto para o resto da vida. Populações de células imunitárias, que residam nos tecidos, formam uma interface materno-recém-nascido são primordiais na transferência vertical de imunidade. As células T γδ encontram-se em diversas mucosas, uma da qual é a mucosa do aparelho reprodutor feminino, onde estas perfazem a maioria das células T residentes no tecido. Por outro lado, as células B perfazem uma fração substancial de linfócitos residentes na maioria dos tecidos linfoides e não-linfoides, sendo responsáveis pela produção de anticorpos naturais e adaptativos. No desenvolvimento desta tese, demonstraremos a importância que o sistema imunitário materno tem no sistema imunitário pulmonar das crias. Quando comparámos os pulmões de ratinhos filhos de mães TCRδ$^{+/-}$ com mães TCRδ$^{-/-}$ no estado estacionário, observámos uma tendência do ambiente imunitário para respostas imunitárias do tipo-2 nas cria de mães TCRδ$^{-/-}$, tendo um aumento na concentração de citocinas relacionadas com este tipo de resposta imunológica, nomeadamente IL-33 e IL-4, havendo também um aumento de citocinas pro-inflamatórias como o TNF-α e a IL-1α. Além do mais, os pulmões das crias de mães TCRδ$^{-/-}$ apresentavam um aumento de certas populações de células imunitárias produtoras de citocinas de respostas imunitários tipo-2, IL-13+ ILC2s e IL-5+ mastócitos. Após infetarmos com *Nippostrongylus brasiliensis* os ratinhos de mães TCRδ$^{-/-}$ apresentaram diferenças significativas em populações de células imunitárias no dia 6 pós-infeção (sem haver diferenças no dia 2 pós-infeção), tendo aumento do número total de leucócitos, com um aumento de células que estão associadas com respostas imunitários do tipo-2, nomeadamente macrófagos e DCs que expressam PD-L2. Baseando-nos nos nossos resultados, pusemos a hipótese de que o sistema imunitário materno, pudendo estar a influenciar o sistema imunitário das crias através da transferência de microbiota e/ou anticorpos. Para testar a primeira hipótese, administramos um cocktail de antibióticos aos pais, antes serem estabelecidos os cruzamentos, mantendo-os até ao desmame. Consistente com a hipótese, as diferenças observadas relativamente à expressão de citocinas foram abolidas. Por outro lado, a transferência de anticorpos maternos não demonstrou ter influência neste processo, não existindo diferenças nos níveis das diferentes classes de IgG nas mães TCRδ$^{+/-}$ or TCRδ$^{-/-}$, nem nas suas crias. Contundo, usando um modelo complementar, as crias de mães deficientes em células B apresentaram uma tendência para respostas imunitários do tipo-1, existindo um aumento de citocinas relacionadas com respostas imunitárias do tipo-1, como a IL-12p40 e a IL-12p70, havendo também um aumento células NK e neutrófilos, enquanto macrófagos alveolares e CD11b- DCs estavam diminuídos, quando comparados com crias de mães suficientes em células B. Em suma, os nossos resultados demonstram que o sistema imunitário materno tem um grande impacto no sistema imunitário das crias, onde diferenças na composição da microbiota ou a incapacidade de produzir anticorpos poderão ser causadas por deficiências de células imunitárias, tais como os linfócitos T γδ analisados nesta tese, levando a disfunções no sistema imunitário da descendência.

Palavras-chave: células T γδ; células B; microbiota; anticorpos maternos; interações materno-recém-nascido; interações materno-fetais; imunidade pulmonar

Abstract

During gestation and nursing, different maternal factors may influence the immune system of the offspring, leading to several alterations that will have an impact throughout life. Immune cell populations that reside in tissues forming the maternal-newborn interface are paramount in the vertical transfer of immunity. γδ T cells can populate several mucosal sites, including the female reproductive tract, where they represent the majority of tissue-resident T cells. B cells on the other hand, make up a substantial fraction of resident lymphocytes in almost all lymphoid and non-lymphoid tissues, being responsible for the production of natural and adaptive antibodies. In this thesis we show the importance of the maternal immune system in the pulmonary immune system of the offspring. When comparing the lungs of mice born from TCRδ$^{+/-}$ or TCRδ$^{-/-}$ dams at steady state, we observed a type-2 biased immune environment in the pups born from TCRδ$^{-/-}$ dams, having an increase in type-2 cytokines, such as IL-33 and IL-4, and of pro-inflammatory cytokines, such as TNF-α and IL-1α. Also, the lungs of pups born from TCRδ$^{-/-}$ dams had an increase in cells that produce type-2 cytokines, such as IL-13+ ILC2s and IL-5+ mast cells. Upon *Nippostrongylus brasiliensis* infection mice born from TCRδ$^{-/-}$ dams showed significant differences in lung immune populations at day 6 p.i. (but no differences at day 2 p.i.), displaying a greater number of total leukocytes, with an increase in cells associated with type-2 immune responses, such as eosinophils and PD-L2-expressing macrophages and DCs. Based on these results we hypothesized that the maternal immune system might be influencing the offspring immune system through the transfer of microbiota and antibodies. To test the first hypothesis, we gave a cocktail of antibiotics to the parents prior to the establishment of the breedings and maintained until fostering. Interestingly, the differences previously observed in the levels of pulmonary cytokine were abolished. Maternal transfer of antibodies, on the other hand, seemed to not be playing a role in this process, as both TCRδ$^{+/-}$ or TCRδ$^{-/-}$ dams and their progenies showed no differences in the circulating levels of different IgG subclasses. However, using a complementary model, pups born from B cell-deficient dams showed a bias towards type-1 immune responses, having an increase in the type-1 cytokines IL-12p40 and IL-12p70, with an increase in lung NK cells and neutrophils, while displaying a decrease in alveolar macrophages and CD11b- DCs, when compared to pups born from B cell-sufficient dams. Overall, our results demonstrate the great impact of the maternal immune system on offspring immunity. Thus, immune cell deficiencies, such as γδ T cells, may result in differences in the microbiota composition or the inability to produce antibodies, which in turn leads to dysfunctions in the offspring immune system.

Keywords: γδ T cells; B cells; microbiota; maternal antibodies; maternal-newborn interactions; maternal-fetal interactions; lung immunity

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1 – Introduction

1.1 – Lung immunity

The lungs main function in the organism is the exchange of gases (such as oxygen and carbon dioxide), between the alveoli and the exterior environment. For this process to be the most efficient possible, the lungs possess a large surface area, with approximately 20 m^2 in humans. Consequently, the lungs can be in contact with several substances that can be harmful, such as allergens, virus and bacteria. Thus, the lungs evolved to counter these aggressions, having a capable immune system to respond against pathogens and allergens, without causing extensive tissue damage (DeKruyff, et al, 2014). The pulmonary immune system is composed by various immune cell populations, such as dendritic cells (DCs), innate lymphoid cells (ILCs), macrophages, regulatory T cells (Tregs), γδ T cells, among others.

Having a diverse immune system, composed by different immune cells capable of interacting among themselves, different immune responses can occur. Type-2 immune responses – the most commonly associated immune response with the pulmonary micro-environment – have been implicated in several pathologies, such as, parasitic infection and allergies (Lloyd & Snelgrove, 2018). However, type-2 immune responses are also crucial for maintaining tissue homeostasis; lungs development and homeostasis, particularly, are quite dependent on a basal type-2 immune response, especially during early life. The mechanical stress arising from the first breath induces the pulmonary epithelium cells to produce the alarmin IL-33. *In vivo*, IL-33 acts in several type-2 immune cells, such as ILC2s, T helper 2 (Th2) cells, mast cells, and some subtypes of DCs, activating them (Kleer et al., 2016; Saluzzo et al., 2017; Molofsky, Savage, & Locksley, 2015). Also, upon injury, ILC2s and Th2 secrete IL-4 and IL-13, generating alternatively activated macrophages (M2) due to the expression of IL-4Rα in macrophages. These IL-4Rα+ macrophages, in the presence of apoptotic cells, initiate tissue repair, since M2 are able to secrete collagen and RELMα, molecules that are vital for tissue regeneration and repair (Lloyd & Snelgrove, 2018).

DCs are antigen-presenting cells (APCs), which derive from hematopoietic stem cells in the bone marrow, capable to uptake antigens and polarizing naïve T cells into different effector T cells types (Liu & Nussenzweig, 2010). In rats, it was shown that a subset of these cells migrates to the lung during the fetal life, and are maintained during adulthood (Mccarthy et al, 1992). In mice, DCs numbers increase from birth until weaning and are essential to maintain type-2 biased immune responses, critical for this time period. During the neonatal period, IL-33 produced by epithelium cells is increased due to the mechanical stress induced from first breath, which leads to the production of IL-13 by ILC2s. IL-13, promotes the migration of DCs to the lymph nodes and cytokine production, ultimately leading to a Th2 polarization. In a house dust mite (HDM) allergy model, sensitized neonates present a further increase in the production of IL-33 by epithelium cells and an increase in DCs migrations to the lymph nodes. This increase in DCs migration to the mediastinal lymph nodes, was due to a response to an increase of IL-13 produced by ILC2s upon HDM-sensitization, lead to an increase in the polarization of naïve T cells into Th2 via the increase expression of OX40L in DCs, while also inhibiting the production of IL-12, preventing the polarization of naïve T cells into Th1 (Kleer et al., 2016).

ILCs are a subset of lymphoid cells, that mirror the T helper lymphocytes in cytokine expression and transcriptional programing. These cells are formed in the bone marrow, migrating to the periphery after birth (Stier et al., 2018). One of the subpopulations of ILCs, ILC2s, are particularly enriched in

pulmonary tissue, being implicated in both protective and damaging responses. Upon activation, ILC2s produce type-2 cytokines, such as IL-5 and IL-13 (Stier et al., 2018; Saluzzo et al., 2017). During the first weeks of life, in mice, there is an increase in the numbers of ILC2s in the lung. This increase was dependent of IL-33 production by epithelium cells, due to the stress in the lung at this time period. IL-33 promotes the migration of ILC2s from the bone marrow to the lung, being capable of producing IL-13, which contributes to the polarization of alveolar macrophages (AMs) to a M2 phenotype and activation of DCs (Kleer et al., 2016; Saluzzo et al., 2017; Stier et al., 2018). IL-33 or ST2, the IL-33 receptor, deficiency leads to an increase in the ILC2 progenitors in the bone marrow, and decreased numbers of mature ILC2s in the lung. However, administration of IL-33, overcomes IL-33 deficiency and promote the egress of these cells from the bone marrow to the lungs (Stier et al., 2018).

AMs are a subset of myeloid cells that derive from fetal monocytes progenitors, residing in the alveolar space since the first week of life. These cells are long-lived and have a self-renewal capacity. These cells perform a variety of functions, including aiding in lung development, pathogen clearance and immune homeostasis (Guilliams et al., 2013). AM population expansion in the neonatal lung is coincidental with that from the ILC2 population. ILC2s produce IL-13, which favors the acquisition by AM of an M2 phenotype, characterized by the production of IL-4, IL-13 and/or IL-10. Also, during adult life, IL-13 is needed to maintain resident AMs in the M2 state, being ILC2s also the major source of this cytokine (Saluzzo et al., 2017)

Tregs are a subset of lymphocyte that can be generated in the thymus or in the periphery, being implicated in the regulation of immune responses (Lee & Lee, 2018). These cells are known for their anti-inflammatory capacities (Lee & Lee, 2018) and to induce tolerance (Mosconi et al., 2010), through the production of anti-inflammatory cytokines, mainly IL-10 and TGF-β (Rubtsov et al., 2008). In a study comparing lymphocyte-deficient (Rag-$1^{-/-}$) mice with WT mice upon LPS challenge, it was shown that the resolution of pulmonary inflammation was impaired in Rag-$1^{-/-}$ mice, having a marked neutrophilic and macrophage infiltrations up to day 10 p.i., whereas the WT counterpart had resolved the inflammation at the same time point. To assess if Tregs were mediating the resolution of the acute lung injury provoked by LPS, the authors transferred Tregs from WT mice into Rag-$1^{-/-}$ mice one hour after LPS challenge, having a complete resolution of the inflammation. The resolution was mediated through the production of TGF-β by Tregs, since WT mice that were treated with anti-TGF-β antibody had persistent pulmonary inflammation upon LPS challenge when compared with Rag-$1^{-/-}$ mice that were transferred with IL-$10^{-/-}$ Tregs (D'Alessio et al., 2009). Also, in an allergy mouse model, it was shown the impact of Tregs in the control of airway inflammation. Ovalbumin (OVA)-sensitized mice presented an increase of Th2 cytokine expression in contrast to the controls. To assess the role of Tregs the authors transferred CD4+ CD25+ T cells from the lungs of naïve mice to OVA-challenged mice. These mice, showed a decrease in Th2 cytokine levels, while IL-10 and TGF-β were increased, having a significant reduction of inflammatory cells in the lungs and airway hyperresponsiveness (Joetham et al., 2007).

γδ T cell are a subset of lymphocytes that colonizes mucosal tissues, such as the lung, capable of responding to different pathogens, while also promoting tissue repair (Dejima et al., 2011; Guo et al., 2018). One of their key features is their ability to rapidly produce pro-inflammatory cytokines, such as IFN-γ and IL-17 (Ribot et al., 2009). IFN-γ is mainly associated with type-1 responses, being shown to have an important role in clearing viral infections (Spitaels, Roose, & Saelens, 2016), while IL-17 is implicated in type-17 responses, where a deficiency of this cytokine, impairs the bacterial clearance (Cheng et al., 2012).

Some tissues, including the lungs, are more susceptible to strong type-1 and type-17 responses, being unable to fully recover from the collateral damage and loosing fitness (Matzinger & Kamala, 2011); thus, upon external insults, pulmonary type-2 immune cells respond to eliminate threat, whilst keeping homeostasis. During parasitic infections, for instance, the large size of the parasite causes tissue damage and, as a consequence, the release of alarmins, such as TSLP and IL-33. On one hand, TSLP directly acts on basophils, which in turn produce IL-4, while also inhibiting the production of IL-12, a type-1 cytokine, by DCs (Gause, Wynn, & Allen, 2013). On the other hand, IL-33, can act directly in ILC2s, inducing the production of IL-4 and IL-13, which can polarize macrophages to a M2 phenotype and promoting DCs migration to the lymph nodes and polarizing Th0 into Th2 (Kleer et al., 2016). These alarmins are important for inducing ILC2s to produce IL-4 and IL-13, alongside with eosinophils and basophils producing IL-4 and IL-5. These cytokines, in turn, promote the differentiation of M2 macrophages, which promote wound repair (Gause et al., 2013). In some situations, however, the lungs can employ different immune responses, such as type-17, in a trade-off between enhanced parasite killing at the expense of increased tissue damage (Sutherland et al., 2014). Also, in allergic diseases, such as asthma, uncontrolled type-2 responses can induce pathology. In asthma, where there is an eosinophilic inflammation in the lungs, it was shown an increase of Th2 cells, IgE and type-2 cytokines, such as IL-4, IL-5 or IL-13 (Finn & Bigby, 2009). After an allergic challenge, granulocytes, such as eosinophils and basophils, are recruited to the lungs and release histamine-containing granules, amplifying the hypersensitivity response. These cells can also function as an APC being able to initiate or enhance Th2 responses (Kim, Dekruyff, & Umetsu, 2010). Th2 cells produce IL-4, which stimulates the production of IgE, leading to a mast cell degranulation, eosinophil influx to the airway and increase the airway responsiveness, and IL-5 a cytokine being involved in the production, differentiation, maturation and activation of eosinophils (Kips, 2001).

Type-1 immune responses can also occur in the lungs, for instance during viral infections. Upon Influenza virus infection, DCs present the viral antigen to naïve CD4+ T cells, generating Th1 cells, which produce IFN-γ, TNF-α and IL-2, helping CD8+ cytotoxic T cells and B cells in the virus clearance (Spitaels et al., 2016). Naïve T cells are polarized by IL-12, differentiating into Th1, making this cytokine associated with type-1 immune responses (Heufler et al., 1996). Also, it was shown that depletion of DCs decreases the numbers of CD8+ T cells upon influenza infection causing a higher mortality in mice. This result was then reverted upon reconstitution of DCs in an MHC I dependent manner, with a significant increase in the IFN-γ+ CD8+ T cells (Mcgill, Rooijen, & Legge, 2008).

In order to constrain overreactive pro-inflammatory responses, in the lung there are mechanisms that promote an anti-inflammatory response. Some macrophages, such as the AM, have anti-inflammatory capabilities. When an inflammatory process is in course, AM phagocyte apoptotic cells to prevent the release of pro-inflammatory substances into the environment (Allard, Panariti, & Martin, 2018). Also, in a study where the susceptibility of AM-deficient and -sufficient mice to *Streptococcus pneumoniae* infection was compared, it was shown that AM-deficient mice present a large immune infiltrate with destruction of the parenchyma, accompanied by an increase of the pro-inflammatory cytokines TNF-α and IL-1β (Knapp et al., 2003). Also, it was shown, in allergic diseases, the inverse correlation between IL-10 levels and severity of the disease (Hawrylowicz & Garra, 2005), while the adopted transfer of OVA peptide-specific Tregs to OVA-sensitized mice reduced airway hyperreactivity, eosinophil recruitment and type-2 cytokine expression, due to the increase of IL-10 expression (Kearley, Barker, Robinson, & Lloyd, 2005).

1.2 – γδ T cells in lungs inflammation

γδ T cell development differ significantly from that of αβ T lymphocytes, since they leave the thymus with full effector capacities, namely cytokine production, without need for peripheral differentiation (Ribot et al., 2009). γδ T cells are generated during the embryonic development in the thymus, being developed in coordinated waves, and migrating posteriorly to different non-lymphoid tissues in the periphery (Carding & Egan, 2002) (Figure 1.1). The main functional feature of γδ T cells is the production of the pro-inflammatory cytokines, IFN-γ and IL-17, separating in two subsets, the IFN-γ- and the IL-17-producers (Ribot et al., 2009). Although found in low numbers among circulating lymphocytes, γδ T cells represent the majority of tissue resident lymphoid cells in barrier sites. Thus, both their developmental program and tissue localization give these cells the capacity to respond fast to extrinsic stimuli (Carding & Egan, 2002). These characteristics of γδ T cells, contribute for their classification as innate-like lymphocytes, bridging the innate and adaptive branches of the immune system.

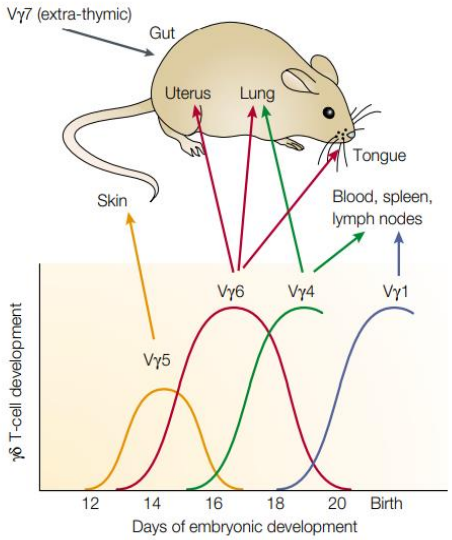

Figure 1.1 – Embryonic development timepoints of γδ T cells and migration.

Adapted from Carding & Egan 2002

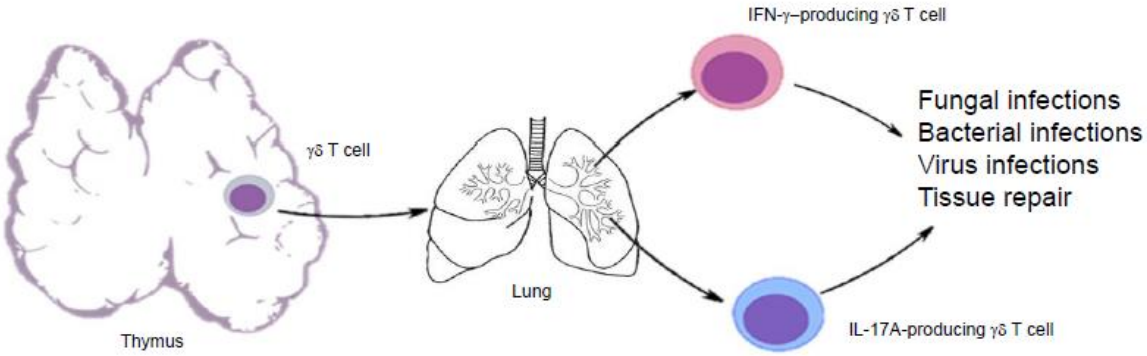

Figure 1.2 – γδ T cells subpopulations and functions in the lungs.

The lungs harbour different populations of γδ T cells (Augustin, Kubo, & Sim, 1989; Born et al., 2000), responsible for the clearance of different fungal (Dejima et al., 2011), helminthic (Sutherland et al., 2014) and viral (Guo et al., 2018)infections, mainly through the production of IFN-γ and IL-17 (Figure 1.2).

In a mouse model of *Staphylococcus aureus* infection γδ T cell-deficient mice display impaired pathogen clearance (Cheng et al., 2012). The authors reported that the role of γδ T cells against *S. aureus* is mediated by the production of IL-17. γδ T cell-deficient mice had reduced expression of this cytokine, in an early stage of infection, resulting in an impaired neutrophil infiltration, since IL-17 is involved in neutrophil migration and activation, thus having a less efficient bacterial clearance (Cheng et al., 2012).

γδ T cell have also been implicated in viral infections (Guo et al., 2018). During Influenza infection in neonates, γδ T cells are the main source of IL-17A. These cells respond rapidly to the infection, as one day after the infection, *Il17a* gene is upregulated, accompanied by the increase in number and frequency of the IL-17A-producing γδ T cell in the lungs. Interestingly, the authors suggested a protective role of IL-17A, since TCR$\delta^{-/-}$ mice showed an increase mortality upon infection when compared to their γδ T cell-sufficient counterparts. To test the protective role of IL-17A, both γδ T cell-deficient and -sufficient animals were infected with Influenza virus and concomitantly a low-level dose of mouse IL-17A was administered, having a positive effect in the survival of TCR$\delta^{-/-}$ mice. This increase of survival was due to an upregulation of IL-33 by the lung epithelial cells, in a IL-17A-dependent fashion. The increase in IL-33 lead to an accumulation of ILC2s and Tregs, mainly amphiregulin producers, which in turn promoted tissue repair responses (Guo et al., 2018).

In a mouse model of *Nippostrongylus brasiliensis* infection, the authors reported an increase of Ym1 and Ym2, two chitinase-like proteins, being associated in with different pathologies. Over-expression of Ym1 in the lungs induces an increase in the number of neutrophils. The authors suggested Ym1 induced the recruitment of neutrophils by up-regulating the expression of the cytokine IL-17A, with γδ T cells being the main producers of this cytokine. In fact, Ym1 and Ym2 overexpression led to an increase in total number of γδ T cells and IL-17A-producing γδ T cells. The mechanism behind the IL-17A production of γδ T cells by Ym1, was due to an increase of IL-1β. When the IL-1 receptor was

blocked neutrophil accumulation, and IL-17 production from γδ T cells was significantly decrease. When Ym1 was blocked during *Nippostrongylus brasiliensis* infection, tissue destruction was reduced, with a decrease also in IL-17A production and neutrophilia. However, the blockade of Ym1 or depletion of IL-17A lead to an increase of worm burden in the gut. With these findings the authors suggested, Ym1 enhances the parasite control, with the trade-off of increase pulmonary damage (Sutherland et al., 2014).

During allergic pathologies, it was shown the capacity of γδ T cells in regulating the airway inflammation (Jaffar et al., 2011; Kinyanjui et al., 2013; Murdoch, Gregory, & Lloyd, 2014). In an OVA-induced mouse model of asthma, prolonged OVA challenge led to an increase in γδ T cells, accompanied by the increase of Th2 cells and eosinophils, which decreased overtime, while γδ T cells remained elevated. To understand the contribution of γδ T cells in the airway remodeling, the authors depleted γδT cells after the inflammation was established. The γδ T cell-depleted mice presented an increased eosinophilia in the pulmonary tissue. The mechanism by which γδ T cells were suppressing the observed type-2 response was through the decrease of IL-4 and IL-5, these cytokines being increased in γδ T cell-depleted mice upon OVA challenge. (Murdoch et al., 2014). Furthermore, in an IL-13-induced airway disease, the authors treated mice with IL-13 and purified from the lungs γδ T cells. After isolation, the cells were transferred to naïve mice, where the total inflammatory cell influx decreased, with the reduction of eosinophils and IL-17 producing lymphocytes, thus regulating allergic airway disease (Kinyanjui et al., 2013)

Although γδ T cells have been shown to regulate airway inflammation during allergies, they were also reported to have pro-inflammatory functions in these pathologies (Zheng & Yang, 2014; Zuany-Amorim et al., 1998; Hahn et al., 2004). In an OVA-induced mouse model of asthma, γδ T cell-deficient mice had a decrease in airway inflammation, when compared to littermate controls. This decrease in inflammation was accompanied with a decrease in OVA-specific IgE expression in the serum, while IL-5 and IL-4 expression, eosinophils and T cell infiltrate were decreased in the bronchoalveolar lavage (BAL) fluid. However, upon administration of IL-4 with the first immunization, γδ T cell-deficient mice presented similar levels of OVA-specific IgE in the serum, IL-5 and eosinophils in the BAL fluid, to the littermate controls. This suggest γδ T cell are important the establishment of for a type-2 inflammation in the lung, contributing to the pathogenesis of the disease (Zuany-Amorim et al., 1998). In a study by Hahn and colleagues, it was reported that γδ T cell, specifically Vγ1+ are responsible for promoting airway responsiveness (Hahn et al., 2004). Upon depletion of total γδ T cells and Vγ1+ γδ T cell in OVA-sensitized mice, the authors observed a decrease in the airway hyperresponsiveness, accompanied with a decrease in IL-13 and an increase in IL-10, when compared to WT mice. Furthermore, after adoptive transfer of Vγ1+ γδ T cell to TCR$\delta^{-/-}$ mice, the airway hyperresponsiveness was similar to WT mice, with increased levels of IL-13 and IL-5, while having a decrease the expression of IL-10 in the BAL fluid (Hahn et al., 2004).

In sum, pulmonary γδ T cells have been associated with both highly inflammatory and homeostasis-promoting immune responses. Given these apparently contradictory roles of γδ T cells during lung inflammation, it is yet to be determined if these differences stem from the discrete programming of different subsets of γδ T cells or dependent on the different models and contexts employed.

1.3 – *Nippostrongylus brasiliensis* infection

N.b. is a helminth that infects rodents, whose primary site of infection is the gut, although, throughout its life cycle passes through other organs. The eggs of the helminth are passed in the feces, hatching

after 24h, being able to infect after 3 to 4 days. The larvae start the infection in the skin, penetrating it until reaching the blood stream. After this, the larvae migrate to the lungs, staying there for 2-3 days. The larvae start to migrate from the alveoli to the trachea, from where they are able to reach the esophagus, to then gain access to the gastrointestinal tract, where they maturate and become sexually competent; with the release of new eggs a in the feces, the cycle repeats (Swain et al., 2016).

Upon N.b. infection, the lungs develop a marked emphysema, with the destruction of the alveolar wall and the enlargement of the air space, which leads to an increase of lungs size. Due to the destruction N.b. causes in the lungs, microhemorrhages can be observed within the air space (Marsland, Kurrer, Reissmann, Harris, & Kopf, 2008). The peak of the pulmonary damage, associated with N.b. infection, occurs at approximately day 2 post-infection., when the emphysema and hemorrhage are more pronounced, resolving over time. On the other hand, the peak of the inflammatory response is at approximately day 6 post-infection, evidenced by the maximum expression of type-2 cytokines, mainly IL-13 and IL-4 (Chen et al., 2012) (Figure 1.3).

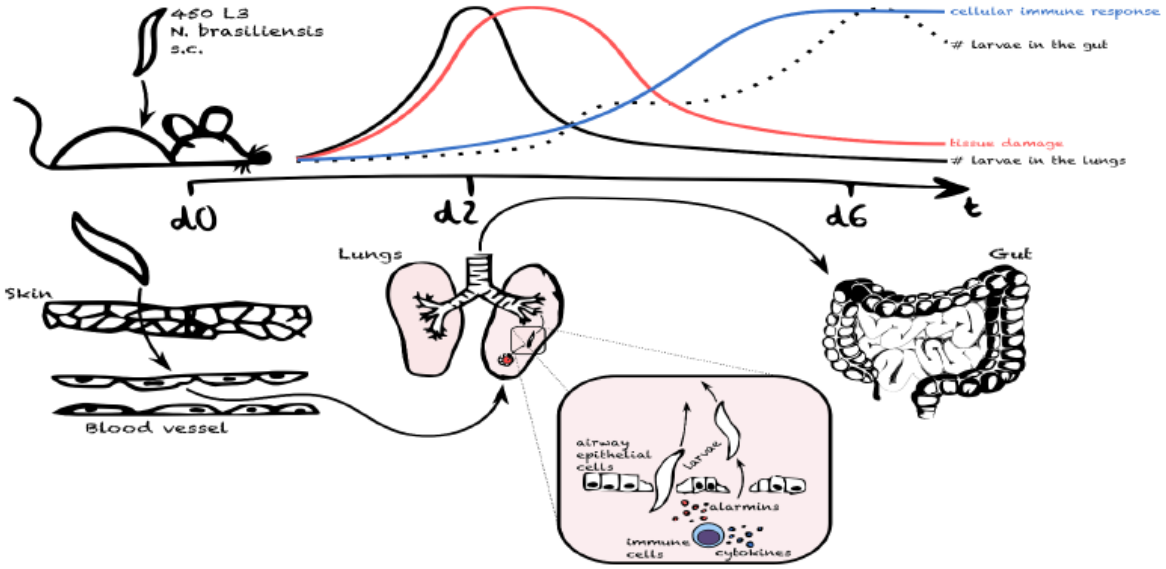

Figure 1.3 – N.b. infection model

Figure by Pedro Papotto

N.b. induces mainly a strong type-2 immune response in the lungs (Hung et al., 2013;Balic, Harcus, Holland, & Maizels, 2004; Voehringer, Shinkai, Locksley, & Francisco, 2004; Thawer et al., 2014). The increase of IL-33 induced by N.b., leads to a rapid increase of the number of IL-13-producing ILC2s in WT mice. IL-13, in turn, promoted the expression of RELMβ, a cytokine that drives worm expulsion, while increasing eosinophil recruitment to the lungs (Hung et al., 2013). Also, it was shown the proteins secreted by N.b. are able to stimulate DCs, which in turn upregulate OX40L, inducing Th2 immune response with the increase of IL-4 (Adam Balic Eur. J. Immunol. 2004). On the other hand, it has been shown that, upon N.b. infection, mice lacking PD-L2, presented a strong Th2 response, with a significant increase of IL-4, IL-5 and IL-13, resulting in eosinophilia and hyper-IgE, suggesting a role for PD-L2 in the inhibition of Th2 immune responses (Ishiwata et al., 2010). In fact, PD-L2 expressing macrophages were shown to be induced by IL-4. Highlighting this pathway as a way of type-2 immune responses to be self-restrained (Loke & Allison, 2003).

1.4 – Preliminary data

Our current project arose from previous observations in our group, when studying the role of γδ T cells during lungs injury. In order to assess how γδ T cells could impact in the repair responses after N.b. infection, we were comparing lungs damage in adult γδ T cell-deficient mice with C57BL/6J (γδ T cell-sufficient) mice, as controls for the genetic background.

Interestingly, γδ T cell-deficient mice exhibited an increase in pulmonary damage at day 2 p.i (Figure 1.4A-C). Similarly, at day 6 p.i. the lungs of γδ T cells-deficient mice also presented an increase in pulmonary damage (Figure 1.4D-F). These results suggested that γδ T cells in mice had a protective role upon pulmonary injury.

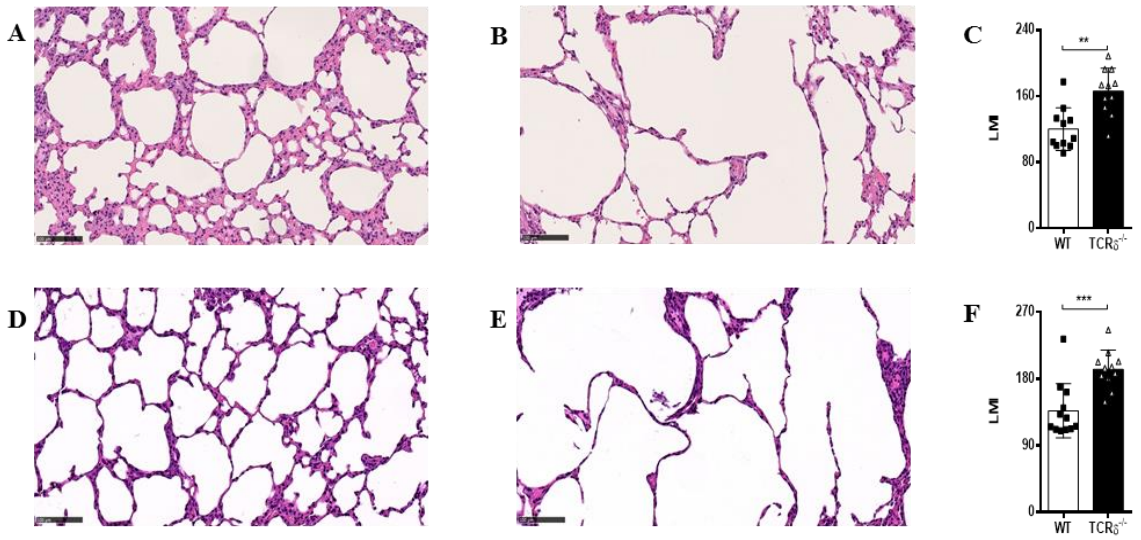

Figure 1.4 – Pulmonary damage for WT vs TCRδ$^{-/-}$ mice upon N.b. infection at day 2 and day 6 p.i.

(A) Lungs slice of a WT mice at day 2 p.i. **(B)** Lungs slice of a TCRδ$^{-/-}$ mice at day 2 p.i. **(C)** Quantification of pulmonary emphysema at day 2 p.i. **(D**) Lungs slice of a WT mice at day 6 p.i. **(E)** Lungs slice of a TCRδ$^{-/-}$ mice at day 6 p.i **(F)** Quantification of pulmonary emphysema at day 6 p.i. **(C)** n=11 mice per group. Data pooled from two independent experiments. **(E)** n=11-12 mice per group. Data pooled from two independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using a Mann-Whitney test. * - $p < 0,05$; ** - $p < 0,01$; *** - $p < 0, 001$; **** - $p < 0,0001$.

Although the differences were clear, we changed our approach in order to achieve a refinement in our experimental design. Instead of using as controls mice from the same strain as the one employed to generate our knockout strain, we began to use littermate controls (LMCs), in order to have a normalized genetic background and, mainly, to have a better control of environmental factors that might influence our results. It has been shown, that after backcrossing a gene of interest to a mouse strain, a linked chromosomal fragment is also transferred, which in turn can affect other protein structures and expression, altering the effect of the target gene, influencing the experimental results, while the LMCs are genetically identical, except for the target gene. Furthermore, the use of LMCs abrogates different environmental cues, since both experimental groups are born from the same mother and kept in the same cage, being in the same conditions (Holmdahl & Malissen, 2012). Using LMCs, TCRδ$^{+/+}$ mice (Figure 1.5A) with TCRδ$^{-/-}$ mice (Figure 1.5B) presented no differences regarding tissue damage after N.b. infection (Figure 1.5C).

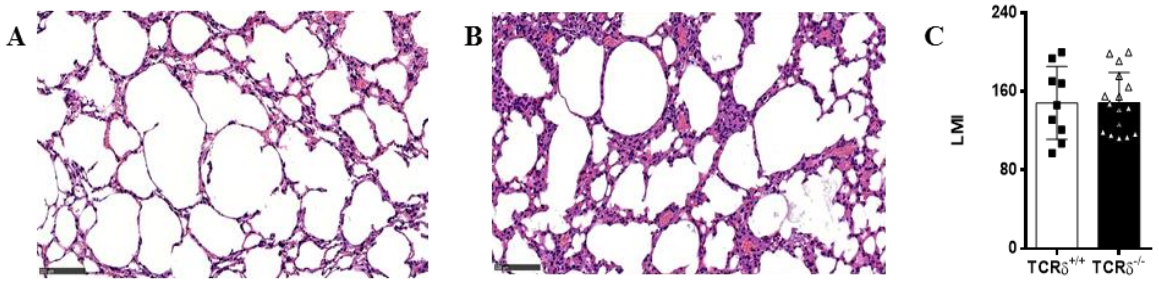

Figure 1.5 – Pulmonary damage of LMCs upon N.b. Infection at day 2 p.i.

(A) Lungs slide from a TCRδ$^{+/+}$ mice. **(B)** Lungs slide from a TCRδ$^{-/-}$ mice. **(C)** Quantification of pulmonary emphysema at day 6 p.i. n=9-16 per group. Data pooled from two independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using a Mann-Whitney test

Based on these contrasting results, one hypothesis that could explain our observations is that the mothers were the cause of this difference, as in the first set of experiments, the knockout mice were coming from a knockout line, in where both parents were missing the TCRδ gene, being compared to a separate line of WT mice, where both parents had both copies of the TCRδ gene (Figure 1.6A), while for the littermate controls both parents had one copy of the TCRδ (Figure 1.6B). Having two separate lines, could lead to several changes, derived not only from genetic drifting, but also from environmental cues that could be altering the immune response (Holmdahl & Malissen, 2012). On the other hand, LMCs are genetically identical, and also are exposed to the same environmental pressures, since both groups were raised together in the same cage (Holmdahl & Malissen, 2012).

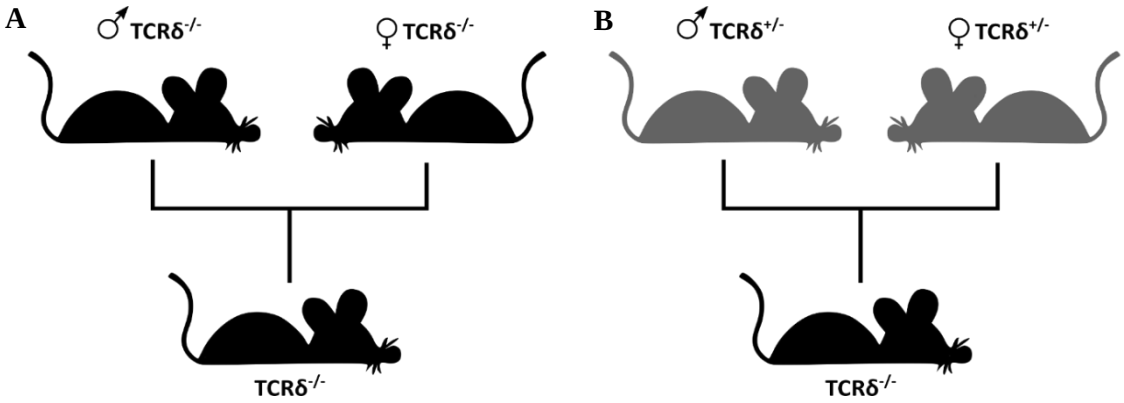

Figure 1.6 – Different breedings for the TCRδ$^{-/-}$ mice and LMCs

(A) Breeding for the KO mice. **(B)** Breeding for the LMC mice. Figure by Pedro Papotto

The way the mothers could be having an impact in the immune system of the offspring could be done by various methods. Our hypothesis focused in two mechanisms. The first was a modulation according to the maternal microbiota, while the second was the existence of a modulation of the offspring immune system through maternal antibodies.

1.5 – Maternal-Fetal interactions

Throughout pregnancy, the fetus is constantly receiving products from the maternal metabolism and habits (Wu, Bazer, Cudd, Meininger, & Spencer, 2004). These maternal products, normally, prevent a detrimental fetal development, providing the nutrients and signals necessary for a normal development.

During pregnancy, not only the various fetal organ systems are being formed, but also the fetal immune system is undergoing development and maturation (Holt & Jones, 2000). Some factors which have a great impact in the fetal immune development are the maternal microbiota and maternal antibodies.

1.5.1 – Maternal microbiota

In the last few years, the microbiota has been thoroughly studied. The microbiota is composed by a collection of bacteria, fungi, virus, archaea and protists, which are in an intimate relation with their host (Baquero & Nombela, 2012; Parfrey, Walters, & Knight, 2011; Hoffmann et al., 2013; Castro-nallar et al., 2015). Some authors even consider the microbiota an indenpendent organ for having an active metabolism, and affecting the physiology of other systems, such as the immune system (Baquero & Nombela, 2012; Hara & Shanahan, 2006). Initially, it was thought that the microbiota was exclusively present in the gut, but several studies pointed to several other organs, where commensal bacteria could be found, such as the lungs, skin and vagina (The Human Microbiome Project Consortium, 2012).

The microbiota has been implicated in several pathologies; and alterations in the microbiota composition (dysbiosis) contributes to diseases, such as asthma (Nyangahu & Jaspan, 2019; Trompette et al., 2014) type-1 diabetes (Nyangahu & Jaspan, 2019; Wang et al., 2018), or inflammatory bowel disease (Nyangahu & Jaspan, 2019; SARTOR, 2010). Additionally, the microbiota from one specific tissue can affect distal organs, and, for example, alterations in gut microbiota can contribute for the development of chronical pulmonary diseases (Marsland, Trompette, & Gollwitzer, 2015). In fact, the existence of a gut-lung axis, a crosstalk between the gut and lung, has gained a lot of attention in the past few years (Marsland et al., 2015). In chronical pulmonary diseases, such as asthma, there has been shown a correlation between gut microbiota modifications and disease development (Fujimura & Lynch, 2015; Rivas, Crother, Arditi, & Angeles, 2016; Chua et al., 2018). There are evidences that the increase in a *Ruminococcus* species in the intestinal microbiota, was associated with the development of asthma or allergic rhinitis, as children with these pathologies, showed an increase of this bacterial species. To assess if *Ruminococcus* species had a potential for inducing asthma, the authors developed a mouse model in which *Ruminococcus gnavus* population was expanded; these mice presented an airway hyper-responsiveness and inflammation, accompanied by an increase in type-2 cytokines, such as IL-33 by the colon tissue, and type-2 immune cells, such as ILC2s and eosinophils (Chua et al., 2018).

One of the mechanisms of microbiota impact in the immunity is through microbial products (Blacher, Levy, & Tatirovsky, 2017; Marsland et al., 2015; Smith et al., 2013). Recent studies have shown the fermentation product of dietary fibers, such as short chain fatty acids (SCFA), can have a protective role in allergic airway inflammation, having anti-inflammatory properties, such as inducing Treg or altering DCs function (Marsland et al., 2015). In germ-free mice (GF), SCFA concentration in the colon was decreased, accompanied with a decrease of colonic Tregs (cTregs) numbers. Upon administration of SCFA to the mice for three weeks, the frequencies and numbers of CD4+ T cells, specifically in cTregs. When assessing the effect of SCFA in the suppressive capacity of cTregs, the authors treated germ-free mice with propionate, and isolated cTregs stimulating *in vitro* with propionate, assessing the expression of Foxp3 and IL-10. Both treatments significantly increased the expression, although, the in vitro treatment did not increase the production of TGF-β, suggesting that SCFA contributes to the increase, specifically, of Foxp3+ IL-10-producing cTregs (Smith et al., 2013). Furthermore, butyrate, another SCFA, induces changes in DCs, by suppressing the expression of of IL-12, IL-6 and Relb, contributing for the Treg differentiation. Also, butyrate promotes a modulation in immune responses in macrophages,

by inhibiting pro-inflammatory factors, such as *Il6*, *Nos2* and *Il12*, contributing for the tolerance towards the commensal bacteria (Blacher et al., 2017).

Maternal microbiota can also have an impact in the immune system of the offspring, as depending on the delivery mode, the microbiota of the offspring suffers alterations, ultimately leading to a dysregulation of the immune system (Macpherson, Agüero, & Ganal-vonarburg, 2017; Bokulich et al., 2016; Hansen et al., 2014). In humans, the changes provoked by the delivery mode have an impact in the maturation and diversity of the microbiota during the first two years of life. It was shown, that the microbiota of both cesarean-section (c-sec) and vaginally born infants have a similar degree of maturation during the first six months, having similar balance of *Bacteroides*, *Bifidobacterium*, and *Enterobacteriaceae*. However, by the first year, c-sec infants had an abundance of *Clostridiales* and *Enterobacteriaceae*, while vaginally delivered infants presented an abundance of *Firmicutes* and *Clostridiales* (Bokulich et al., 2016). In a study, when comparing c-sec delivered mice with vaginal delivered mice, an increase was found of *Prevotella* genus members and the *Ruminococcaceae* family in the gut microbiome of c-sec born mice. In addition to the observed dysbiosis, the immune system presented a decrease of activated T regs in the mesenteric lymph nodes (MLN) and IL-10 levels in the serum (Zachariassen et al., 2018). Also, the delivery mode has an impact in tolerogenic immunity by altering the microbiota. The microbiota of c-sec delivered NOD mice presented an increase *Rikenellaceae* and *Clostridiaceae* families while vaginally delivered NOD mice presented an increase *Oscillospira* and *Ruminococcus* genera. These alterations in the microbiota lead to a decrease in tolerogenic immune response in the c-sec delivered mice. In a study comparing the spleen and MLN of c-sec delivered with vaginally delivered mice, the authors found an increase in Tregs and tolerogenic CD103+ DCs from vaginally delivered mice, while the expression of *Il-10* was decreased in mice born via c-sec (Hansen et al., 2014).

1.5.2 – Maternal antibodies

Antibodies or immunoglobulins (Ig) are proteins secreted mainly by a subset of B cells, the plasma cells (Ribatti, 2017). These antibodies have different subtypes IgA, IgG, IgM, IgE and IgD all being implicated with immune responses and defense (Schroeder Jr & Cavacin, 2010). IgA is mainly found in mucosal surfaces and circulation, being implicated in the defense against inhaled and ingested pathogens in the mucosa (Woof & Kerr, 2004). On the other hand, IgG and IgM are mainly found in circulation, while IgG is a a trigger for effector mechanisms and can neutralize virus particles and toxins (Vidarsson, Dekkers, & Rispens, 2014), IgM is the first line of defense against pathogens, and can inhibit IgG autoantibody formation (Lobo, 2016). Finally, IgE is found in circulation and has been implicated as a trigger of allergic responses, although having an important role against helminthic infections (Oettgen, 2016). Although IgD is found in the plasma, the functions of this immunoglobulin are unclear, and it is not known to play a role in major effector mechanisms, however, IgD can bind to *Moraxella catarrhalis* proteins resulting in B cell stimulation and activation (Schroeder Jr & Cavacin, 2010).

Immunoglobulins have a wide range of interactions with immune cells, binding to cell surface receptors, activating the immune cells, while certain immune cells promote the secretion of specific immunoglobulin isotypes (Kok, Bestebroer, & Strijp, 2014; Stone, Prussin, & Metcalfe, 2010; Rezende et al., 2018). Neutrophils, for instance, are capable of binding different subclasses of IgG and IgA. In the case of a *Staphylococcus aureus* infection, neutrophils recognize opsonized bacteria, through the Fc receptors FcγRII and FcγRIIIB in the cell surface, being able to phagocyte the bacteria, clearing the infection (Kok et al., 2014). Also, mast cells have been reported to be activated by IgE. Mast cells have been implicated in allergic diseases when activated through FcεR1, which binds to IgE, leading to a

degranulation, releasing histamine, exacerbating the allergic reaction (Stone et al., 2010). Furthermore, there are evidences of γδ T cells having an impact in immunoglobulin production. In a study comparing TCRδ$^{-/-}$ with TCRδ$^{+/+}$ mice, it was shown that in the intestinal lamina propria, TCRδ$^{-/-}$ mice had impaired numbers of IgA-producing plasma cells, leading to a reduction of total IgA in the serum, saliva, feces and bile. Moreover, upon oral administration of tetanus toxoid and cholera toxin, the TCRδ$^{-/-}$ mice presented a significant decrease, for both toxins, in IgA response, with a decrease in the IgA-producing cells and IgA production (Fujihashi et al., 1996). More recently, the induction of T follicular helper cells (Tfh) differentiation by γδ T cells (Rezende et al., 2018) could provide a possible mechanism explaining the impaired immunoglobulin production in γδ T cell-deficient mice. In fact, TCRδ$^{-/-}$ mice present a decrease in the self-reactive IgG and IgM in the serum, when compared to wt mice. Furthermore, upon immunization with Complete Freund's Adjuvant, TCRδ$^{-/-}$ mice display a reduction of Tfh cells in draining LN, with these cells expressing less CD40L, suggesting these cells do not provide an adequate signal to B cells to produce antibodies (Rezende et al., 2018).

The first interactions of immunoglobulins with the organism is during pregnancy and breastfeeding, where different subtypes of immunoglobulins are transferred to the offspring, trough the placenta and milk (Jennewein, Abu-raya, Jiang, & Alter, 2017; Weaver, Arthur, Bunn, & Thomas, 1998; Ohsaki et al., 2018; Mosconi et al., 2010). In the uterus, IgG is mainly transported by neonatal Fc receptor (FcRn), from the placenta to the fetal bloodstream (Jennewein et al., 2017), while IgA is secreted to the milk in the mammary glands and passed to the baby during breastfeeding (Weaver et al., 1998). Maternal antibodies play major roles in the modulation of the offspring immune system. In a study, mice born from antibody deficient mice had an increase in effector T cells in the MLN and Peyer's patches that respond to antigens derived from commensal bacteria. Thus, being needed to promote tolerance to commensal bacteria by dampening T cell-dependent immunity (Koch et al., 2016). Also, maternal IgG is needed to stimulate the B cell lineage. It has been shown there is an increase in the bone marrow, of B and pre-B cells, when comparing mice that were nursed by a dam that produced IgG with one that did not (Malanchère, Huetz, & Coutinho, 1997)

Maternal antibodies also have been implicated in promoting tolerance and preventing allergies, through the induction of Tregs by OVA-IgG-ICs (Ohsaki et al., 2018; Mosconi et al., 2010). Mice born from dams that were not sensitized with OVA developed a food-allergic reaction, when sensitized with OVA. These mice presented an allergic phenotype, with an increase of OVA-specific IgE production, accompanied with the increase of IL-4, frequencies and numbers of mast cells and *Il13* mRNA expression in the intestine, when compared with pups born from dams sensitized with OVA (Ohsaki et al., 2018). Furthermore, in an OVA-inducing asthma model, mice breastfed from unsensitized mothers presented an increase of eosinophilic airway inflammation, with increased levels of OVA-specific IgE and lungs IL-13 and IL-5 secretion (Mosconi et al., 2010). Also, pups born from OVA-sensitized mothers had an increase in numbers and frequencies of Tregs in the MLN, which upon OVA challenge, these mice exhibited a higher level of expansion and proliferation then pups born from unsensitized mothers. The mechanism behind the promotion of tolerance was through allergy-specific IgG-IC that are transferred via breast milk. Mothers sensitized with OVA presented an increase of OVA-specific IgGs and IgE in breast milk. Consistently, the offspring of OVA-sensitized dams presented an increase of OVA-IgG-IC, in the serum. This IgG-IC is processed and presented by CD11c+ DCs in the MLN, that express FcRn, to CD4+ T cells, differentiating into Tregs, thus, promoting tolerance in the offspring (Mosconi et al., 2010; Ohsaki et al., 2018).

1.6 – Hypothesis and Objectives

The hypothesis of this thesis is that the maternal immune system controls offspring immunity by shaping the tissue-resident immune cell populations and cytokine production. In order to assess that, we have:

1. Analyzed the immune cell populations in the lung in steady state conditions and during N.b. infection, in pups born from γδ T cell-deficient and -sufficient dams.
2. Analyzed the immune cell populations in the lung in steady state conditions in pups born from B cell-deficient and -sufficient dams.
3. Determined the inflammatory response and tissue damage upon N.b. infection, in pups born from γδ T cell-deficient and -sufficient dams.
4. Determined the impact of maternal transfer of antibodies and microbiota in the offspring.
5. Quantified cytokine production in pups born from γδ T cell- and B cell-deficient dams.
6. Quantify gene expression of genes associated with lung homeostasis of pups born from γδ T cell-deficient and -sufficient dams.

2 – Methods

2.1 – Animals

The mice used where adults 6-12 weeks of age, and pups with 14 days of age. C57BL/6J were purchased from Charles River Laboratories. C57BL/6J.TCRδ$^{-/-}$ (referred as TCRδ$^{-/-}$) mice were obtained from The Jackson Laboratory. C57BL/6J.JHT$^{-/-}$ (referred as JHT$^{-/-}$) mice were obtained from Instituto Gulbenkian de Ciência (Oeiras, Portugal). Mice were bred and maintained in the specific pathogen-free animal facilities of Instituto de Medicina Molecular (Lisbon, Portugal). All animal experiments were done in compliance with the relevant laws and institutional guidelines and approved by local and European ethics committees.

2.2 – Infection Model

Nippostrongylus brasiliensis was maintained by serial passage through Sprague-Dawley rats, as previously described (Lawrence, 1996). Third-stage larvae (L3) were washed ten times with sterile PBS prior to subcutaneous injection of 450 L3 per adult mice, and 300 L3 per young (21 days of age) mice. At day 2 and day 6 post infection (p.i), the mice were sacrificed, and their lungs were collected and processed for further analysis.

2.3 – Histopathology Analyses

Lungs samples were fixed in 10% neutral buffered formalin and processed for paraffin embedding, sectioned and stained with Hematoxylin-Eosin. Each slide was digitalized using a NanoZoomer-SQ Digital slide scanner. To quantify emphysema like damage, we used the Linear means intercepts (LMI) method. After digitalization of each lungs slide, between 10 and 20 non-overlapping pictures, of each lung, were taken. The pictures were analyzed using ImageJ software. For each picture, 6 horizontal lines were drawn, as shown in Figure 2.1, and counted each time a line intercepts an alveolar. For the LMI calculation, the length of one line was multiplied by the number of pictures taken and the number of lines in one picture, and then divided by the total number of intersections of the pictures taken.

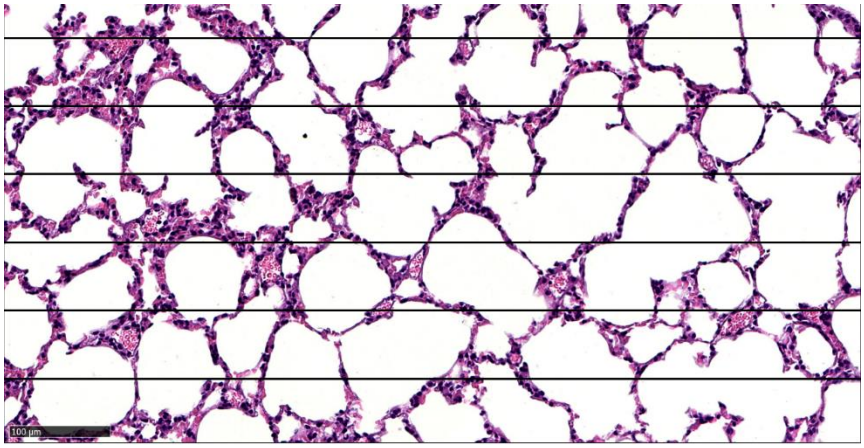

Figure 2.1 – Methodology for the LMI calculation

2.4 – Immune phenotyping

2.4.1 – Isolation of lung Leukocytes

After harvesting the lungs, these were cut into small pieces, put in 10 mL of digestion medium (DNase I (Sigma-Aldrich) 100 µL:10 mL of RPMI 1640 with 5% of Fetal Bovine Serum (FBS) and Collagenase type IV (Roche) 30 µL:10 mL of RPMI-5%FBS) and left in a shaker for 45 min at 37ºC. The lungs pieces were mashed in a 40 µm cell strainer. The cell strainer was cleaned with RPMI-5%FBS until 20 mL. For the isolation of the leukocytes, a 33% percoll (Sigma-Aldrich) gradient was prepared. First, a solution of 40% percoll (1,33 mL percoll 90% + 1,67 mL RPMI-5%FBS per sample) was mixed with the sample and a 70% percoll solution (1,4 mL percoll 90% + 0,6 mL RPMI-5%FBS per sample) was posteriorly added. The samples proceeded to a centrifugation (2400 rpm, 30 minutes, 23ºC, 0 acceleration and 0 break). A ring in the interface was formed, extracted (approx. 2 mL) and added 1mL of RBC lysis buffer (1:10 in water). After 5 minutes, RPMI-5%FBS was added to the samples, until 5 mL. A final centrifugation (1500 rpm, 5 minutes, Room temperature) was made, the supernatant discarded, and the sample is resuspended in RPMI-5%FBS for further analysis.

2.4.2 – Isolation of Spleen Leukocytes

After harvesting the spleen, the organ was mashed in a 40 µm cell strainer. The cell strainer was cleaned with RPMI-5%FBS until 20 mL. 1mL of RBC lysis buffer (1:10 in water) was added to the sample. After 5 minutes, RPMI-5%FBS was added to the samples, until 5 mL. After centrifugation (1500 rpm, 5 minutes, Room temperature) the sample is resuspended in RPMI-5%FBS for further analysis.

2.4.3 – Staining of Immune Cells

For the surface staining, after isolating leukocytes, we transferred the samples to a 96-well V-bottom plate. A centrifugation is executed (1800 rpm, 3 minutes, Room temperature), the supernatant discarded and 200 µL of FACS Buffer (PBS-1x + 1% FBS) added for cleaning. The samples were resuspended in 50 µL of the Antibody mix comprised of saturated concentrations of the mAb, listed below, plus anti-CD16/CD32 (eBioscience) and incubated for 20 minutes in the fridge. After incubation, 200 µL of FACS Buffer were added to the samples and a final centrifugation (1800 rpm, 3 minutes, Room temperature) is made. The samples were resuspended with 200 µL of FACS Buffer and passed to FACS tubes. The wells were washed with 50 µL of FACS Buffer and put in the FACS tubes.

For intracellular cytokine staining, cells were stimulated with PMA (phorbol 12-myristate 13-acetate) (50ng/mL) and ionomycin (1µg/mL), in the presence of Brefeldin A (10µg/mL) (all from Sigma) for 3h at 37°C. Cells were stained for the identified cell surface markers, fixed 30min at 4°C and permeabilized with the Foxp3/Transcription Factor Staining Buffer set (eBioscience) in the presence of anti-CD16/CD32 for 10 min at 4°C, and finally incubated for 1h at room temperature with identified above cytokine-specific Abs in permeabilization buffer.

The single stainings were prepared according to the number of colors used, we put in a FACS tube 1 drop of BD™ CompBeads, one for the positive and one for the negative per 5 colors used. 100 µL of FACS Buffer were added per color to the tube. 100 µL of the solution were put into separate tubes, one per color, and added 0,5 µL of an antibody. The beads incubated for 10 minutes, after which 200-400 µL of FACS Buffer were added to each tube.

All the samples and single stainings were analyzed on FACS Fortessa (BD Bioscience) and FlowJo (Tree Star).

The list of antibodies used can be seen in Table 2.1, and the gating strategies for the cell identification in Figure 7.1-7.4.

Table 2.1 – List of antibodies used

Antibody	Clone	Company
CD24	M1/69	BioLegend
CD11c	N418	BioLegend
MHC II	M5/114.15.2	BioLegend
MHCII	M5/114.15.2	eBioscience
Ly6G	1A8	BD
Siglecf	E50-2440	BD
LIVE/DEAD Fixable Near-IR		Invitrogen
CD64	X54-5/701	BioLegend
Ly6C	HK1.4	BioLegend
CD19	6D5	BioLegend
Gr-1	RB6-8C5	BioLegend
CD23	B3B4	BioLegend
SCA-1	D7	BioLegend
ST 2	DIH9	BioLegend
KLRG1	2F1/KLRG1	BioLegend
Thy1.2	53-2.1	BioLegend
IL-13	eBio13A	eBioscience
CD45	30-F11	BioLegend
CD11b	M1/70	BioLegend
CD8	5H10-1	BioLegend
γδ	eBioGL3	eBioscience
CD3	145-2C11	eBioscience
CD3	17A2	BioLegend
NK1.1	PK136	BioLegend
CD4	GK1.5	eBioscience
CD4	GK1.5	BD
IL-9	RM9A4	BioLegend
IL-5	TRFK5	PharMingen
CD206	CO68C2	BioLegend
PD-L2	TY25	BioLegend
PD-L1	10F.9G2	BioLegend

2.5 – Cytokine profiling

For the cytokine profiling we used two kits from BioLegend®, LEGENDplex™ Mouse cytokine Panel 2 (13-plex) and LEGENDplex™ Mouse Th1/Th2 Panel (8-plex), both having the same protocol.

The lungs from mice were collected and placed in a tube with 1 mm of diameter silica beads (BioSpec Products) and a solution of 1 mL PBS-1x with a cocktail of protease inhibitors (cOmplete ULTRA tablets, Mini; Roche). The tubes were in a mini bead beater to disrupt the lungs. After disruption the lungs were centrifuged (10000 rpm, 10 minutes at room temperature) and the supernatant collected. The samples were stored at -80ºC until usage.

The beads were vortexed for 30 to 60 seconds to resuspend the beads. A 1x Wash Buffer was prepared, by diluting 25 mL of 20x Wash Buffer in 475 mL of deionized water. A lyophilized Matrix was reconstituted, by adding 5 mL of LEGENDplex™ Assay Buffer, waiting 15 minutes and Vortexed to mix well.

8 standards were prepared, where the top standard, C7, was prepared by reconstituting the lyophilized Mouse Cytokine Standard Cocktail with 250 µL Assay Buffer into a tube. 6 other tubes are marked from C6 to C1. 75 µL of Assay Buffer is added to each tube. A 1:4 dilution of the top standard is done, by transferring 25 µL of C7 to C6 tube and mixed well. The other standards are prepared from a serial 1:4 dilution, same as the previous, obtaining C5, C4, C3, C2 and C1. Assay Buffer was used as the 0 pg/mL (C0) as seen on Table 2.2.

Table 2.2 – Table for the standard dilution in the LEGENDplex™ Mouse cytokine Panel 2 protocol

Tube/ Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (pg/mL) *	Final Conc. (pg/mL) **
C7	--	--	--	10,000	50,000
C6	1:4	75	25 µL of C7	2,500	12,500
C5	1:16	75	25 µL of C6	625	3,125
C4	1:64	75	25 µL of C5	156.3	781.3
C3	1:256	75	25 µL of C4	39.1	195.3
C2	1:1024	75	25 µL of C3	9.8	48.8
C1	1:4096	75	25 µL of C2	2.4	12.2
C0	--	75	--	0	0

***Top standard concentration of IL-1α, IL-1β, IL-3, IL-12p40 , GM-CSF, IL-12p70, is 10 ng/mL; **Top standard concentration of IL-7, IL-11, IL-23, IL-27, IL-33, IFN-β, and TSLP is 50 ng/mL.**

The assay was performed in a 96-well V-bottom plate. Various solutions were added to the plate, represented in Table 2.3.

Table 2.3 – Solutions added to the plate, corresponding volume and well

Solution	Volume (µL)	Well
Matrix C	12,5	Standard
Assay Buffer	12,5	Sample
Standards	12,5	Standard
Sample	12,5	Sample
Mixed Beads	12,5	All
Detection Antibodies	12,5	All

The plate was covered with aluminum foil and left in a shaker for 2 hours at 600 rpm room temperature. Without washing 12,5 µL of SA-PE was added to each well. The plate was covered with aluminum foil and left for 30 minutes at the same conditions as before. After this a centrifugation is done at 1000 g for 5 minutes. The supernatant removed and 200 µL of 1x Wash Buffer was added to each well, vortexed for 1 minute and centrifuged at 1000 g for 5 minutes. The supernatant was removed and 200 µL of 1x Wash Buffer was added to the wells, and the solution passed to FACS Tubes. The samples were read on a flow cytometer. The data is analyzed in LEGENDplex™ Data Analysis Software.

Cytokine concentrations were normalized using total protein concentration in tissue homogenate, determined in a NanoDrop spectrophotometer (ThermoFisher).

2.6 – Antibodies analysis

For the quantification of immunoglobulins, we used a kit from BioLegend®, LEGENDplex™ Mouse Immunoglobulin Isotyping Panel. For this blood was collected from the cheek pouch in adults, and from decapitation in pups. The serum samples were stored at -20ºC until usage.

Firstly, the beads were put in a vortex for 30 to 60 seconds to resuspend the beads. A 1x Wash Buffer was prepared, by diluting 25 mL of 20x Wash Buffer in 475 mL of deionized water.

Secondly, 8 standards weree prepared. The top standard, C7, was prepared by reconstituting the lyophilized Mouse Immunoglobulin Isotyping-panel Standard Cocktail with 250 µL Assay Buffer into a tube. 6 other tubes were marked from C6 to C1. 75 µL of Assay Buffer added to each tube and a 1:4 dilution of the top standard was done, by transferring 25 µL of C7 to C6 tube and mixed well. The other standards were prepared from a serial 1:4 dilution, same as the previous, obtaining C5, C4, C3, C2 and C1. Assay Buffer was used as the 0 pg/mL (C0) as seen on Table 2.4

Table 2.4 – Table for the standard dilution in the LEGENDplex™ Mouse Immunoglobulin Isotyping protocol

Tube/Standard ID	Serial Dilution	Assay Buffer to add (µL)	Standard to add	Final Conc. (ng/mL)
C7	--	--	--	6400
C6	1:4	75	25 µL of C7	1600
C5	1:16	75	25 µL of C6	400
C4	1:64	75	25 µL of C5	100
C3	1:256	75	25 µL of C4	25
C2	1:1024	75	25 µL of C3	6.25
C1	1:4096	75	25 µL of C2	1.56
C0	--	75	--	0

The essay was performed in a 96-well V-bottom plate. We added 25 µL of Assay Buffer in every well and 25 µL of the sample to each sample well, and 25 µL of each standard to the respective standard well. The beads were vortexed for 30 seconds and added 25 µL of the mixed beads to each well. The plate was covered with aluminum foil and left in a shaker for 2 hours at 800 rpm room temperature. After this we centrifuged the plate (250 g, 5 minutes, room temperature) and discarded the supernatant and added 200 µL of washing buffer to each well. We then centrifuged the plate (250 g, 5 minutes, room temperature) and discarded the supernatant and added 25 µL of detection antibodies to each well. The plate was covered with aluminum foil and left in a shaker for 1 hours at 800 rpm room temperature. Without washing 12,5 µL of SA-PE was added to each well. The plate was covered with aluminum foil and left for 30 minutes at the same conditions as before. After this a centrifugation is done at 1000 g for 5 minutes. The supernatant was removed and 200 µL of 1x Wash Buffer added to each well, vortexed for 1 minute and centrifuged at 1000 g for 5 minutes. The supernatant is removed and 200 µL of 1x Wash Buffer were added to the wells, and the solution is passed to FACS Tubes. The samples were read on a flow cytometer. The data was analyzed in LEGENDplex™ Data Analysis Software.

2.7 – Gene expression

2.7.1 – RNA extraction

For the RNA extraction, RNeasy® Mini Kit from Qiagen® was used. After collecting and weighting the lungs, the organs were put in a tube with silica beads and a solution of 1 mL RLT Buffer. The samples were put in a bead beater for 45 seconds. This step was repeated one time. After disruption of the organs, the samples were centrifuged for 10 minutes at 10000 rpm at 4º, the supernatant was collected to RNAse free tubes and the tubes are stored at -80º.

1 volume of 70% Ethanol is added to the lysate (if sample is 500 µL add 500 µL of Ethanol) and mixed by pipetting. 700 µL of the sample were transferred to a RNeasy Mini spin column with a 2 mL collection tube, centrifuged for 15s at 13000 rpm at 4ºC and the flow-through discarded. 700 µL of RW1 are added to the spin column, centrifuged for 15s at 13000 rpm at 4ºC and the flow-through discarded. 500 µL of RPE are added to the spin column, centrifuged for 15s at 13000 rpm at 4ºC and the flow-through discarded. 500 µL of RPE are added to the spin column, centrifuged for 2 min at 13000 rpm at

4ºC and the flow-through discarded. A centrifugation at full speed for 1 min was done in order to dry the membrane. The spin column was placed in a new collection tube and 30-50 µL of RNase-free water were added and the column was centrifuged for 1 min at 13000 rpm. This step was repeated one time. After the extraction, the samples are stored at -80º.

2.7.2 – cDNA conversion and quantification

RNA samples were converted into cDNA. First the reagents are prepared according to Table 2.5. The volume of sample plus reagent is 40 µL. To choose the volume of sample used, we measured the concentration of Nucleic Acid in ng/µL, using Nanodrop 2000™. The volume of sample we added was the amount of volume needed to have approximately 2500 ng of RNA per sample. After this the reagents are added until 40 µL.

Table 2.5 – Reagents for cDNA conversion per sample

Solution	Volume (µL)	Company
Random Primers	4	Invitrogen
5x First-Strand Buffer	16	Promega
dNTPs	3	Abiosystems
RNaseOUT	1,25	Invitrogen
M-MLV	3	Promega
H2O	12,75	

The quantification of cDNA species was performed on an ABI ViiA7 cycler (Applied Biosystems) relatively to endogenous references, such as β2-microglobulin or β-actin. For this we prepared the reagents according Table 2.6. The C_T of each gene was subtracted from the C_T of the endogenous refences and the relative amount was calculated as $2^{-\Delta CT}$.

Table 2.6 – Reagents for cDNA quantification

Solution	Volume (ul/2xwell sample)	Company
SYBR Green	10	Abiosystems
Primer B	0,4	
Primer C	0,4	
H2O	5,2	
Total	16	
Sample	4	

The list of primers we used to assess the different genes are listed in Table 2.7

Table 2.7 – List of primers used

Gene	Primer forward	Primer reverse
β2-Microglobulin	CTGCAGAGTTAAGCATGCCAGTAT	ATCACATGTCTCGATCCCAGTAGA
β-Actin	CGTGAAAAGATGACCCAGATCA	TGGTACGACCAGAGGCATACAG
Sftpa	GATTCTGCAAACAATGGGAGT	TGGTATCAAAGTTGACTGC
Sftpb	TGGCTAGACAGGCAAAAGTG	GGCCTCACATACGCCAAGG
Sftpc	GCATCGTTGTGTATGACTACCAG	AAGCCTCAAGACTAGGGATGC
Sftpd	TTGCCTTCTCCCACTATCAGA	ATCTCCTGGGCATCCTCAA
Spdef	CCCACCTGGACATCTGGA	GACGAGTCCACCTCACCATC
Muc5a	AGGACAGTGGGGCCATTT	TCAAAATAGATCATGGGTGCAT
Muc5b	ATGGTGTGGCCAGCAGAG	CTTGTGGATGAGGATATGGAGTC

2.8 – Antibiotic treatment

TCRδ$^{-/-}$ and TCRδ$^{+/-}$ female and male adult mice were maintained with of a mixture of antibiotics (5mg/ml of streptomycin, 1 mg/ml of ampicilin, 1 mg/ml of collistin, and 0.5 mg/ml of vancomycin; Sigma-Aldrich) in the drinking water for at least three weeks prior the start of the breedings, and then crossed as already described. Antibiotic treatment was kept for the whole duration of pregnancy and fostering.

2.9 – Statistical analysis

GraphPad Prism software was used to perform statistical analysis. We used a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test, if the samples followed a normal distribution, or Mann-Whitney test, if the samples the samples did not followed a normal distribution. The results are presented as p-values: * - $p < 0,05$; ** - $p < 0,01$; *** - $p < 0, 001$; **** - $p < 0,0001$.

2.10 – WT vs LMC

A search was performed in PubMed data base, using the keywords "gamma delta T cells AND infection" and gamma-delta T cells AND infection" and the papers selected according to the criteria of using a mouse model of WT mice vs TCRδ$^{-/-}$ mice or use of LMCs.

3 – Results

3.1 – Damage control by γδ T cells during lungs injury

3.1.1 – The lungs immune environment and cell populations of pups born from TCRδ-deficient dams is biased towards type-2 immune responses

In order to assess the impact of maternal γδ T cells on the offspring immune system, we crossed TCRδ$^{-/-}$ dams with males from the opposite genotype, and *vice-versa* (Figures 3.1A), having offspring that can either be γδ T cell-deficient or γδ T cell-sufficient. First, we collected the lungs from 14 days old pups (since at this age the lungs have a strong type-2 immune response, due to the mechanical stress provoked by breathing (Saluzzo et al., 2017), in order to characterize the immune environment of these pups, regarding tissue cytokine levels, resident immune population and expression of homeostatic genes.

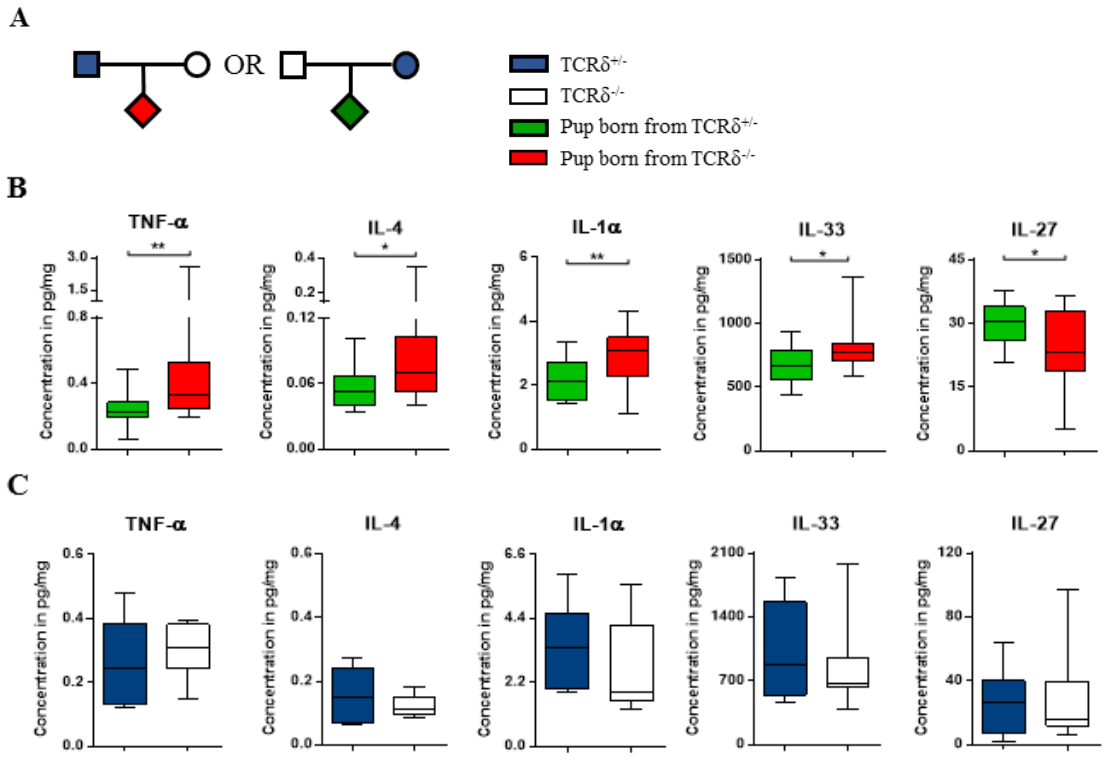

Figure 3.1 – Analysis of the lungs cytokine expression in pups born from TCRδ$^{+/-}$ and TCRδ$^{-/-}$ mothers.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. **(B)** Concentration of cytokines in the lungs, normalized by total protein in the tissue of pups from the breedings described in (A), arranged by maternal genotype. **(C)** Concentration of cytokines in the lungs, normalized by total protein in the tissue of pups from the breedings described in (A), grouped by the offspring genotype. **(B)** n=14-19 mice per group. Data pooled from three independent experiments. **(C)** n=6-11 mice per group Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test * - p < 0,05; ** - p < 0,01; *** - p < 0, 001; **** - p < 0,0001.

Pups born from TCRδ$^{-/-}$ dams displayed increased tissue levels of the type-2 cytokines IL-4 and IL-33, when compared to the progeny of TCRδ$^{+/-}$ dams, whilst presenting decreased levels of IL-27, a cytokine known to inhibit Th2 responses (Yoshimoto, Yoshimoto, Mizuguchi, Nakanishi, & Alerts, 2007). Also,

an increase of the pro-inflammatory cytokines TNF-α and IL-1α was observed in pups born from TCRδ$^{-/-}$ dams (Figure 3.1B). Interestingly, when comparing the cytokine expression according to the genotype of the pups, no differences were found (Figure 3.1C).

Additionally, we also characterized by flow cytometry the different populations of pulmonary resident immune cells in the offspring of TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams (Figure 3.2A). Even though pups born from TCRδ$^{-/-}$ dams present a pulmonary cytokine milieu biased towards type-2 immune responses, we observed no differences in the total numbers of leukocytes (Figure 3.2B) and in the frequencies of type-2 immune cell populations, such as eosinophils, CD11b+ DCs or ILC2s (Figure 3.2C); also, no differences were observed in the distribution of macrophage, monocyte and neutrophil populations (Figure 3.2C). However, in agreement with the increase of tissue levels of type-2 cytokines pups born from TCRδ$^{-/-}$ dams, showed an increase in IL-13-producing ILC2s and IL-5-producing mast cells (Figure 3.2D)

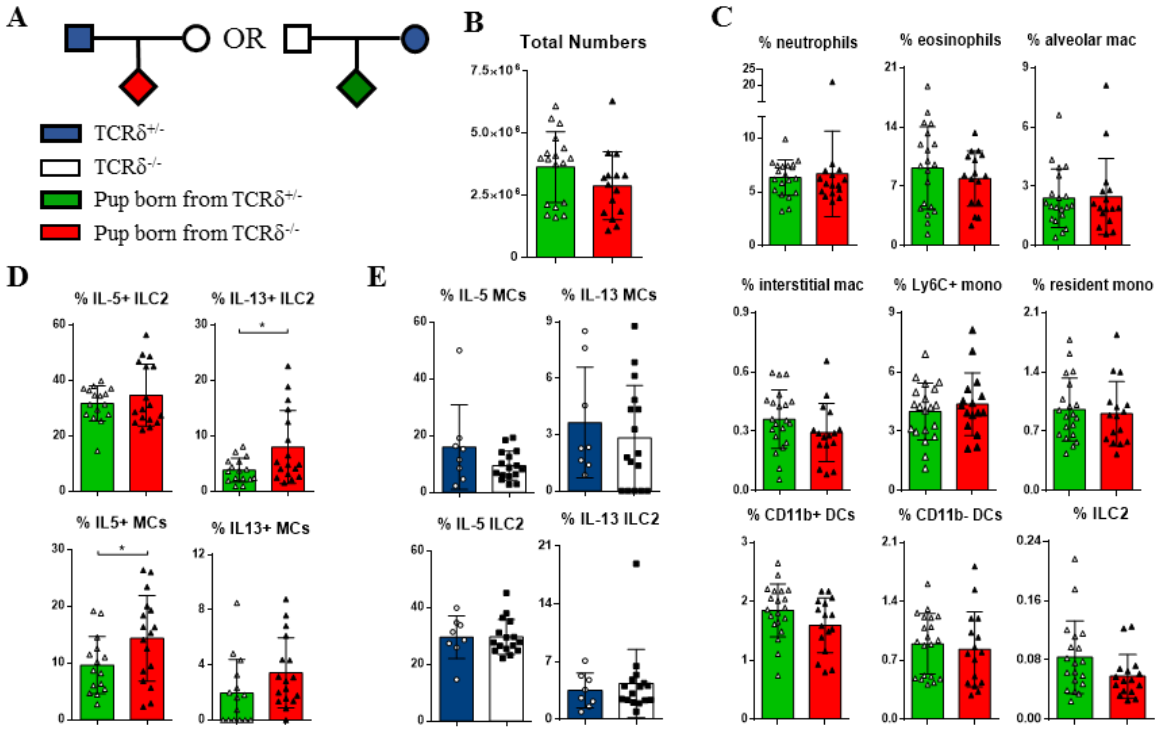

Figure 3.2 – Analysis of the lungs immune cell population in pups born from TCRδ$^{+/-}$ and TCRδ$^{-/-}$ mothers.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. **(B)** Total number of leukocytes (CD45+ cells) in the lungs from pups generated from the breedings described in (A), arranged by the maternal genotype. **(C)** Percentage of immune populations in the lungs within CD45+ cells from pups generated from the breedings described in (A), arranged by the maternal genotype. **(D)** Percentage of ILC2S (defined as Lin-Thy1.2+Sca-1+KLRG-1+cells) and mast cells (defined as Lin-Thy1.2-Sca-1+KLRG-1-cells) producers of type-2 cytokines in the lungs within CD45+ cells from pups generated from the breedings described in (A), arranged by the maternal genotype. **(E)** Percentage of myeloid cells producers of type-2 cytokines in the lungs within CD45+ cells from pups generated from the breedings described in (A), arranged by the offspring genotype. **(B-C)** n=24-27 mice per group. Data pooled from three independent experiments. **(D-E)** n=8-16 mice per group. Data pooled from two independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test * - p < 0,05; ** - p < 0,01; *** - p < 0, 001; **** - p < 0,0001.

Since we observed an increase in tissue type-2 cytokines and type-2 cytokine producing ILC2s and mast cells in the lungs of pups born from TCRδ$^{-/-}$ dams, we analyzed the expression of genes related to

pulmonary homeostasis from the offspring of TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams (Figure 3.3A). Interestingly, the differences in the lungs of pups born from TCRδ$^{-/-}$ dams seems restricted to the pulmonary immune system, as the expression of genes encoding for surfactant proteins and mucins was similar between the progenies of both TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams (Figure 3.3B).

In sum, the absence of maternal γδ T cells leads to an increase in the basal perinatal type-2 inflammation in the lungs of the offspring, evidenced by an increase of tissue levels of IL-4, IL-33, TNF-α and IL-1α, accompanied by an increase in IL-13-producing ILC2s and IL-5-producing mast cells, without the alteration of other immune cell populations

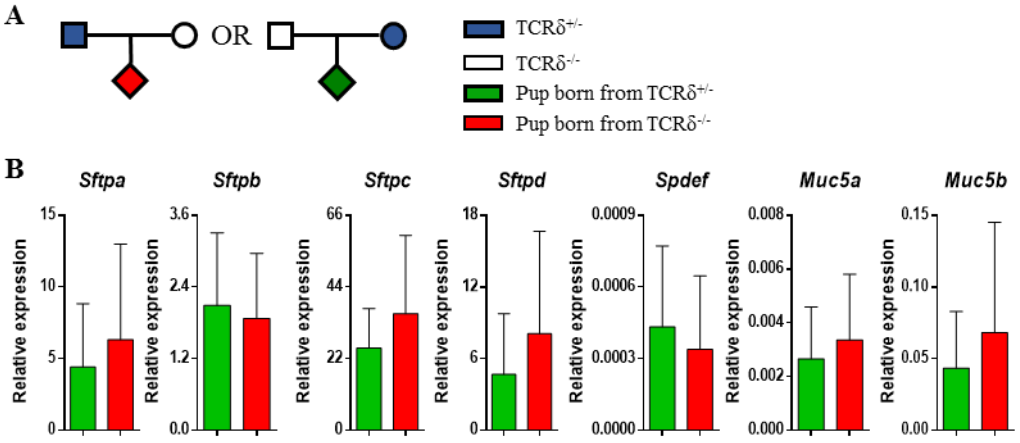

Figure 3.3 – Analysis of the lungs homeostasis genes in pups born from TCRδ$^{+/-}$ and TCRδ$^{-/-}$ mothers.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. **(B)** Relative expression of lungs homeostasis related genes of pups generated from the breedings described in (A), arranged by the maternal genotype. n=14-21 mice per group. Data pooled from two independent experiments Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Mann-Whitney test.

3.1.2 – TCRδ mice have an increase of immune populations related to type-2 immune responses in the lungs upon infection, but not tissue damage

Given that the progeny of γδ-deficient dams had a pulmonary environment biased towards type-2 immune responses in the steady-state, we next sought to understand if these basal differences could affect immunity against invading pathogens. Thus, we infected mice born from TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams soon after weaning with *Nippostrongylus brasiliensis* a helminth known to promote type-2 immune responses in the lungs. We then analyzed the immune response and damage in the lungs at the peak in tissue damage (day 2 p.i.), and at the peak of inflammation (day 6 p.i.).

We collected the lungs of mice born from TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams (Figure 3.4A), and analyzed paraffin-embedded lungs sections, in order to access the pulmonary damage. Injury was determined with the help of the calculation of the Linear Mean Intercept (LMI) value. We found no differences in the LMI at day 2 p.i. (Figure 3.4B) when comparing lungs sections from mice born from TCRδ$^{-/-}$ dams (Figure 3.4C) with the ones from TCRδ$^{+/-}$ dams (Figure 3.4D).

Although in steady state mice born from TCRδ$^{-/-}$ dams had a type-2 biased immune environment, upon infection we found no differences in the absolute number of leukocytes (Figure 3.4E), type-2 immune cells, such as eosinophils and CD11b+ DCs (Figure 3.4F). Also, no differences were found in numbers of macrophages, neutrophils and monocytes (Figure 3.4F).

Altogether, at day 2 p.i. in a N.b. infection model, both mice born from TCRδ$^{-/-}$ and TCRδ$^{+/-}$ dams have a similar response to the helminth, having no differences in pulmonary damage and in the composition of the immune cell infiltrate.

Since the immune response, during N.b. infection, reaches his peak after 6 days, we analyzed at this time point the composition of immune cells and the pulmonary damage associated with the infection (Figure 3.5A).

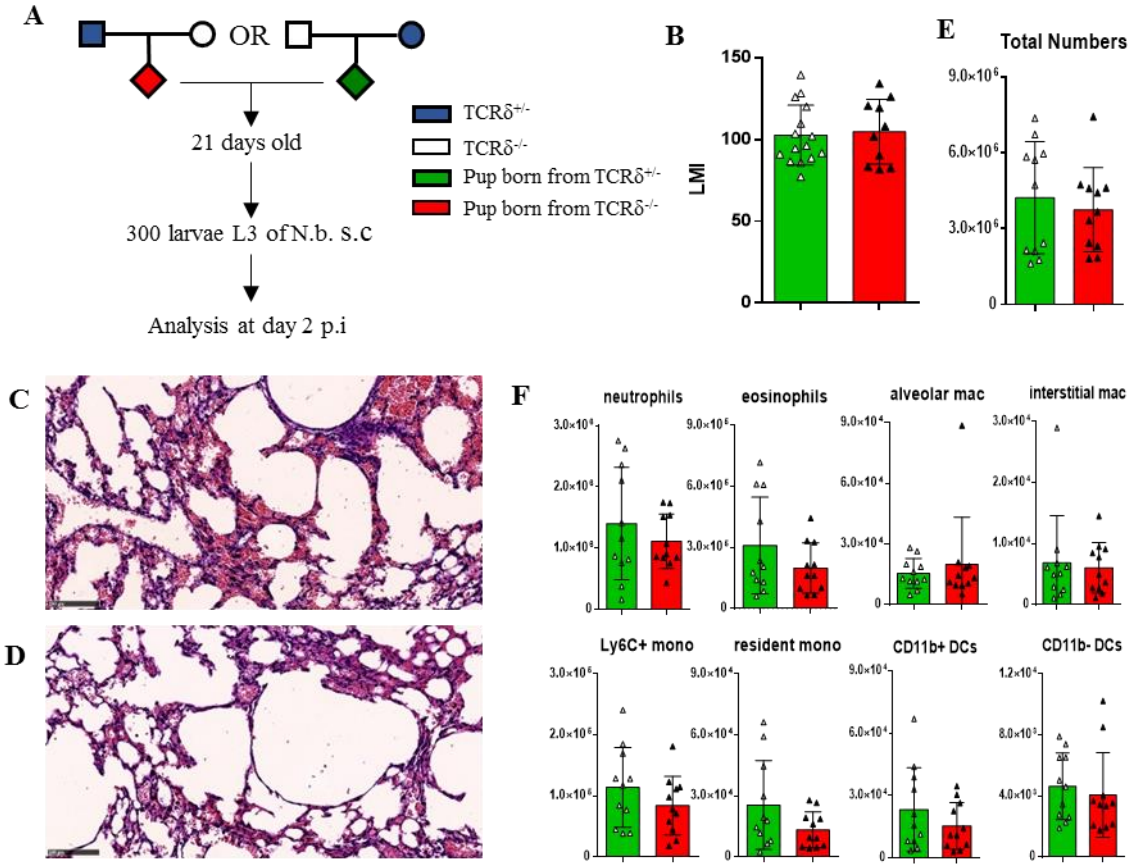

Figure 3.4 – Lungs immune populations and damage at day 2 p.i with N.b. Infection.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. The mice were infected with 300 larvae of N.b and the lungs analysed at day 2 p.i. **(B)** Quantification of pulmonary emphysema at day 2 p.i. from mice generated from the breedings described in (A), arranged by the maternal genotype. 10-20 photos of each lungs were taken in a microscope (x200 Amplification). **(C)** Lungs section from a mouse born from TCRδ$^{+/-}$ mothers. **(D)** Lungs section from a mouse born from TCRδ$^{-/-}$ mothers. **(E)** Total number of leukocytes (CD45+ cells) in the lungs from mice generated from the breedings described in (A), arranged by the maternal genotype. **(F)** Absolute numbers of myeloid cells in the lungs within CD45+ cells from mices generated from the breedings described in (A), arranged by the maternal genotype. **(B)** n= 10-15 mice per group. Data pooled from two independent experiments. **(E-F)** n=11 mice per group. Data pooled from two independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test

In agreement with the data obtained at day 2 p.i. data, we could not find differences in the pulmonary damage (Figure 3.5B) when comparing the lungs of mice born from TCRδ$^{-/-}$ (Figure 3.5C) or TCRδ$^{+/-}$ dams (Figure 3.5D), at day 6 p.i.

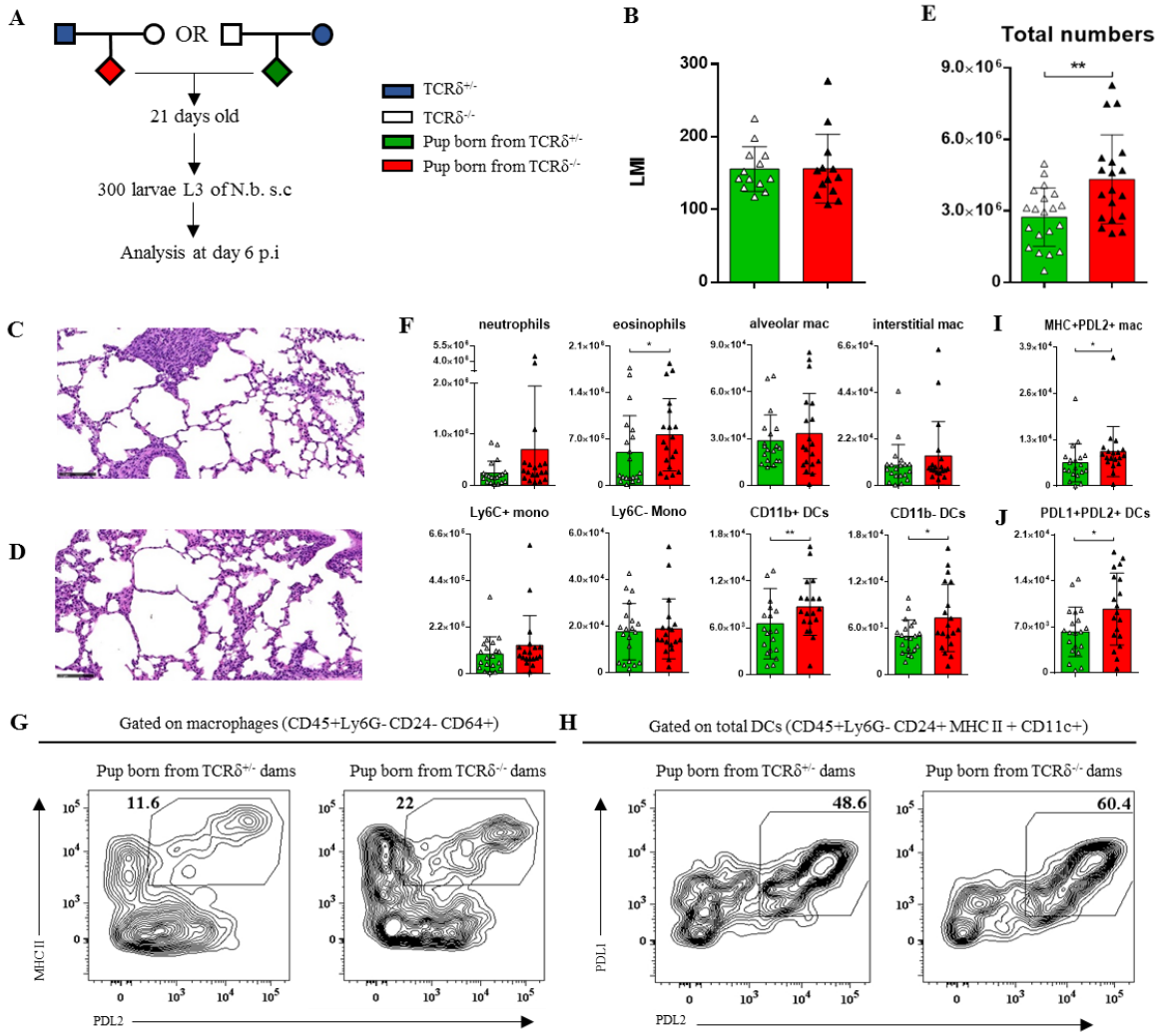

Figure 3.5 – Lungs immune populations and damage at day 6 p.i with N.b. Infection.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. The mice were infected with 300 larvae of N.b and the lungs analysed at day 6 p.i. **(B)** Quantification of pulmonary emphysema at day 2 p.i. from pups generated from the breedings described in (A), arranged by the maternal genotype. 10-20 photos of each lungs were taken in a microscope (x200 Amplification). **(C)** Lungs section from a mouse born from TCRδ$^{+/-}$ mothers. **(D)** Lungs section from a mouse born from TCRδ$^{-/-}$ mothers. **(E)** Total number of leukocytes (CD45+ cells) in the lungs. **(F)** Absolute numbers of myeloid cells in the lungs within CD45+ cells. **(G)** Flow cytometry analysis of PD-L2 expression in macrophages (defined as CD45+ Ly6G- CD24- CD64+ cels). **(H)** Flow cytometry analysis of PD-L2 expression in DCs (defined as CD45+ Ly6G- CD24+ MHC II+ CD11c+ cells). **(I)** Absolute numbers of PD-L2 expressing macrophages. **(J)** Absolute numbers of PD-L2 expressing DCs. **(B)** n=13 mice per group. Data pooled from two independent experiments. **(E-J)** n=19-20 mice per group. Mice were generated from the breedings described in (A), arranged by the maternal genotype. Data pooled from three independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test * - $p < 0,05$; ** - $p < 0,01$; *** - $p < 0,001$; **** - $p < 0,0001$.

Although we had no differences in pulmonary damage, interestingly, the total number of leukocytes was increased in mice born from TCRδ$^{-/-}$ dams, suggesting an increase in the inflammatory response (Figure 3.5E). This increase in the immune infiltrate consisted mainly in type-2 immune cells, such as eosinophils and CD11b+ CDs (Figure 3.5F). We found no differences in neutrophils and monocytes (Figure 3.5F). We also analyzed the expression of PD-L2 in macrophages (Figure3.5G) and DCs (Figure 3.5H). PD-L2 expression in DCs and macrophages, is associated with type-2 immune responses (Ishiwata et al., 2010; Loke & Allison, 2003) interestingly, PD-L2+ DCs and macrophages were increased in absolute numbers in mice born from TCRδ$^{-/-}$ dams (Figure 3.5I and J).

From the data for day 2 and day 6 p.i., we conclude that both mice from TCRδ$^{+/-}$ and TCRδ$^{-/-}$ dams, do not have differences in pulmonary damage. Although at day 6 p.i., in contrast to day 2 p.i., mice born from TCRδ$^{-/-}$ dams sustain a greater immune response to the pathogen. This greater immune response is characterized by an increased immune infiltrate and, accordingly to the data from steady state, a bias to type-2 responses, where we can highlight the increase of eosinophils, CD11b+ DCs and PD-L2-expressing DCs and macrophages

3.1.3 – Maternal microbiota, rather than maternal antibodies, alters the immune system of the progeny of γδ T cell-deficient mice

In order to understand how the lack of maternal γδ T cells could induce the observed type-2 bias in the offspring pulmonary immune system, we next assessed the possible mechanisms of maternal influence, namely transfer of antibodies and microbiota.

To determine if maternal microbiota was having an impact in the maturation of offspring pulmonary immune system, we maintained TCRδ $^{-/-}$ and TCRδ $^{+/-}$ mice (males and females) on an antibiotic cocktail from three weeks prior the establishment of the breeding, throughout the whole length of pregnancy and fostering (Figure 3.6A). Interestingly, after this period of microbiota normalization, the previously observed differences in the cytokine levels were abolished (Figure 3.6B). This result suggests a direct impact in the immune environment of the pups, made by the maternal microbiota.

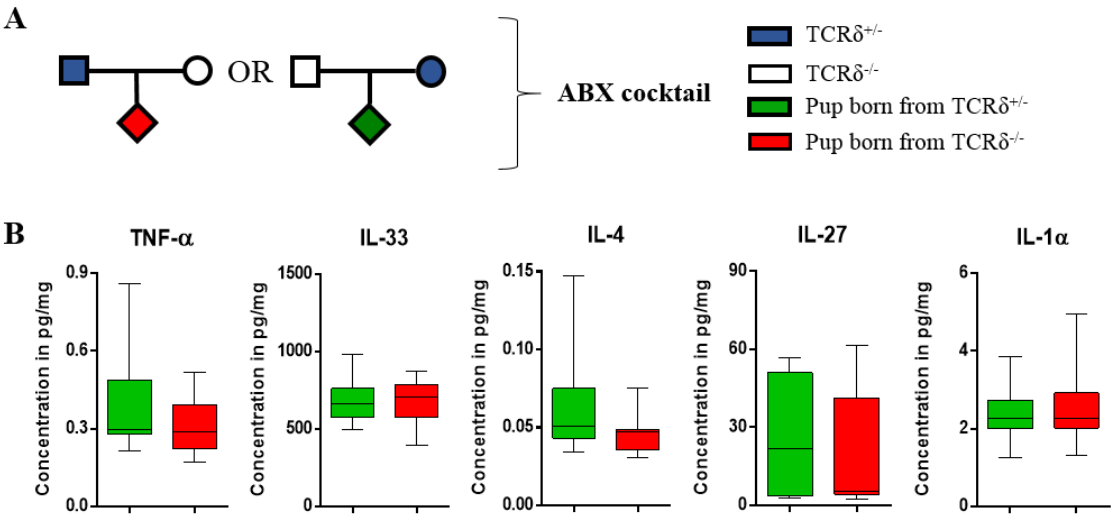

Figure 3.6 – Analysis of the lung environment of pups from mothers that underwent antibiotics.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. An antibiotic cocktail was giving too the parents from three weeks prior to the breedings and maintained throughout pregnancy and fostering. **(B)** Concentration of cytokines in the lungs, normalized by total protein in the tissue of pups from the breedings described in (A), arranged by maternal genotype. n=16-18 mice per group Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test

Since maternal influence can occur through different mechanisms, we next assessed if the impact on the offspring pulmonary immune system was only microbiota dependent, or a synergy between microbiota and maternal antibodies, since it has been shown maternal IgG can transport microbial products from

the placenta to the offspring (Agüero et al., 2016). Thus, in order to determine if absence of γδ T cells could be affecting natural antibody production (Watanabe et al., 2000; Bank, Tanay, Migdal, Book, & Livneh, 1995), and therefore, vertical antibody transfer, we evaluated immunoglobulin levels in TCRδ $^{-/-}$ and TCRδ $^{+/-}$ dams, and their respective progeny, using an ELISA-based assay (Figure 3.7A).

We found no differences in IgG levels between TCRδ $^{-/-}$ and TCRδ $^{+/-}$ dams (Figure 3.7B), suggesting the concentration of IgG in the mothers is not influenced by the absence of γδ T cells. After this, we assessed the concentration of IgG in the pups. Likewise, we could not find any differences in the concentration of IgG subclasses (Figure 3.7C). Accordingly, this suggests that the maternal genotype has no impact in the IgG levels of pups.

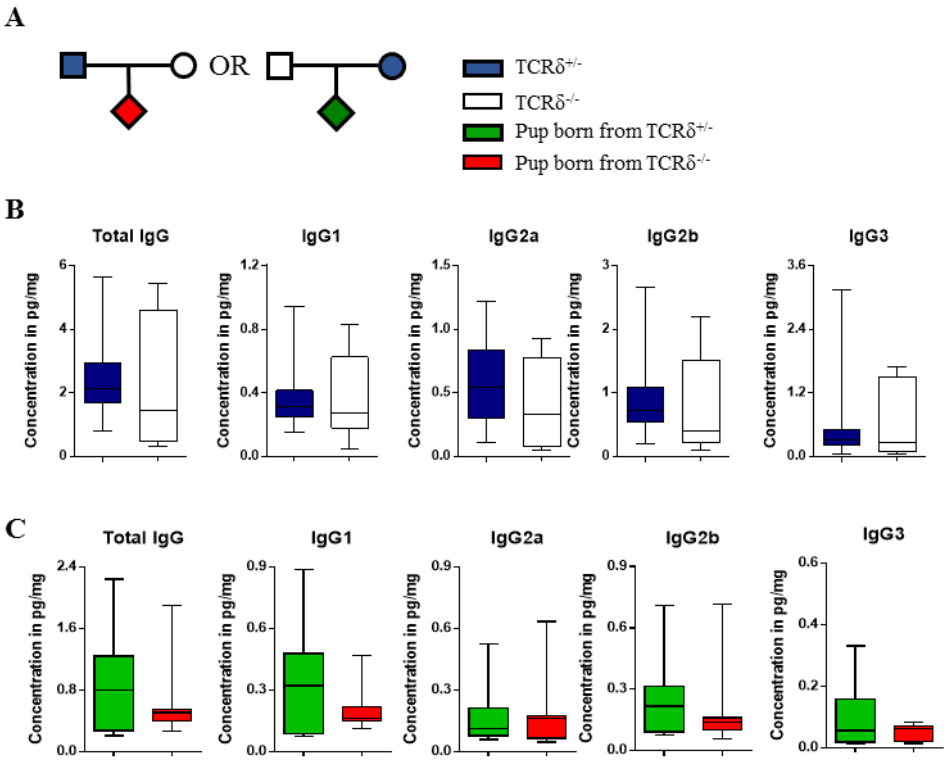

Figure 3.7 – Immunoglobulin levels in the serum of TCRδ deficient and sufficient dams and pups.

(A) Breeding schematics to evaluate the impact of the TCRδ gene in the pulmonary immune system in the offspring. TCRδ$^{-/-}$ dams were crossed with TCRδ$^{+/-}$ males, and vice-versa. **(B)** Concentration of IgG in the serum, normalized by total protein in the tissue of pups from the breedings described in (A), arranged by maternal genotype. **(C)** Concentration of IgG in the serum, normalized by total protein in the tissue of pups from the breedings described in (A), arranged by the offspring genotype. **(B)** n=7-14 mice per group **(C)** n=19-20 mice per group. Data pooled from two independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test

In conclusion, the alterations observed in the pulmonary milieu of the progeny from TCRδ $^{-/-}$ dams seem to be dependent on the maternal microbiota, with maternal antibodies playing no role in the pulmonary immune environment of the offspring

3.2 – The role of maternal antibodies in shaping the immune response

3.2.1 – Pups born from B cell-deficient dams show selective alterations in the cytokine production

Although our previous results suggested that transfer of IgG does not play a role in the alterations we observed in the offspring of γδ T cell-deficient mice, we wanted to assess how the complete absence of maternal antibodies influenced the pulmonary immune system of pups.

Firstly, we compared the tissue cytokine levels in the lungs of pups born from B cell-deficient dams, JHT$^{-/-}$, which do not produce antibodies, with the ones born from wild type mothers, C57BL/6J, which produce antibodies (Figure 3.8A).

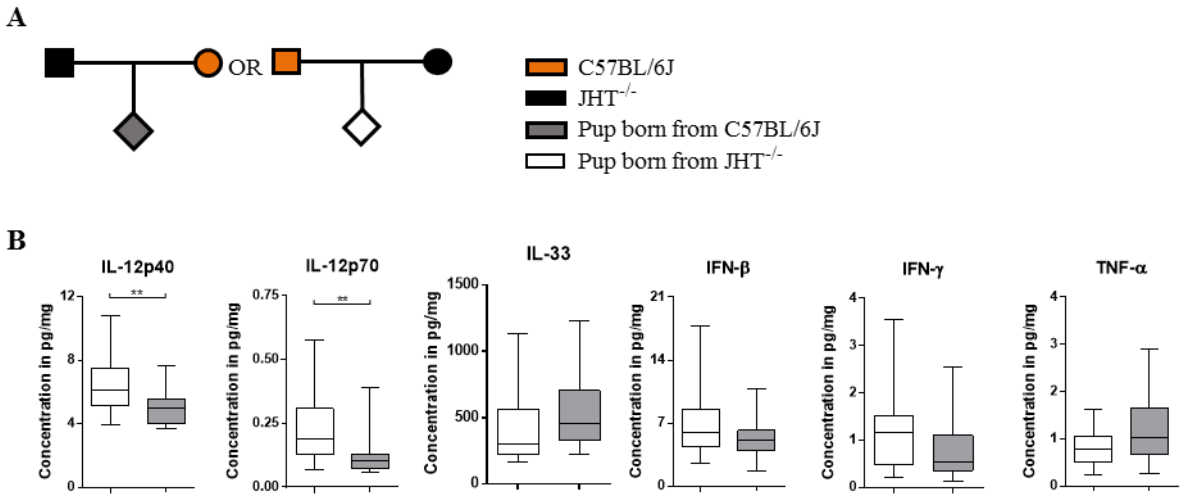

Figure 3.8 – Differences in the cytokine production of pups born from B cell-deficient and -sufficient dams.

(A) Breeding schematics to evaluate the impact of the maternal antibodies in the pulmonary immune system in the offspring. JHT$^{-/-}$ dams were crossed with C57BL/6J males, and vice-versa. **(B)** Concentration of cytokines in the lungs, normalized by total protein in the tissue of pups from the breedings described in (A), arranged by maternal genotype. n=18-22 mice per group. Data pooled from three independent experiments. Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test. * - $p < 0,05$; ** - $p < 0,01$; *** - $p < 0, 001$; **** - $p < 0,0001$.

The lungs of pups from both B cell-deficient and -sufficient dams were collected at day 14 post-birth (Figure 3.8A). The pups were grouped according to the maternal genotype and the cytokine expression was analyzed. The main difference in both groups is in the IL-12 family cytokines (Figure 3.8B), namely IL-12p40 and IL-12p70. These cytokines are associated with the differentiation of naïve T cells into Th1, suggesting a bias towards type-1 immune response in the perinatal lungs of pups from antibody-deficient mothers. As expected, since we have an increase in type-1cytokines, we found no differences in type-2 cytokines, mainly IL-33.

3.2.2 – Pups born from B cell-deficient dams show a type-1 immune bias

Since we had an increase in cytokines associated with type-1 immune responses in the lungs, we wanted to access how this increase was affecting different resident immune cell populations. To this end we analyzed by flow cytometry the lungs of pups born from C57BL/6J and JHT$^{-/-}$ dams (Figure 3.9A).

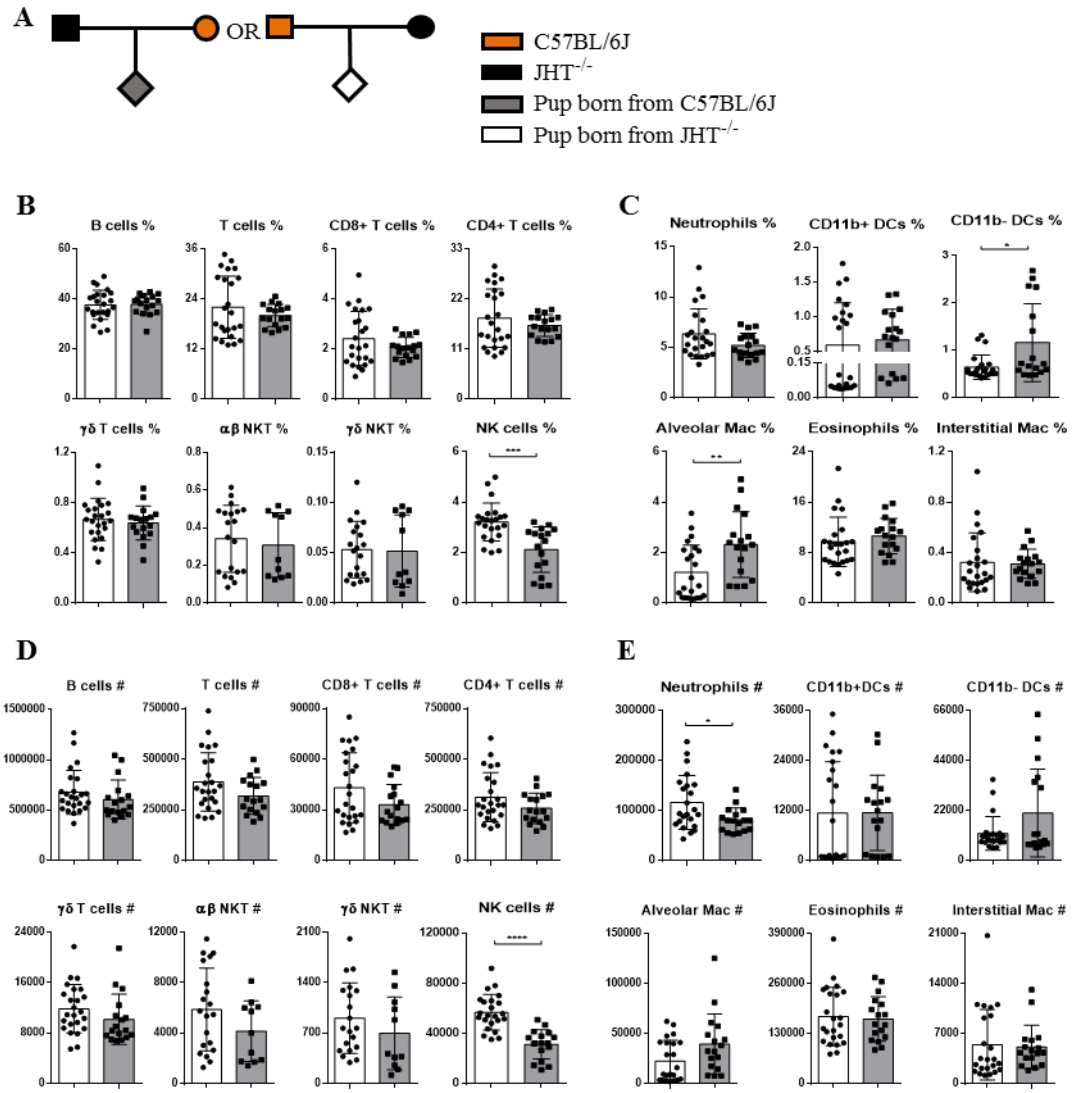

Figure 3.9 – Differences of immune cells in the lungs of pups born from B cells-deficient and -sufficient dams

(A) Breeding schematics to evaluate the impact of the maternal antibodies in the pulmonary immune system in the offspring. JHT$^{-/-}$ dams were crossed with C57BL/6J males, and vice-versa. **(B)** Percentage of lymphoid cells in the lungs of pups. **(C)** Percentage of myeloid cells in the lungs. **(D)** Absolute numbers of lymphoid cells in the lungs of pups. **(E)** Absolute numbers of myeloid cells in the lungs. **(B-E)** n=17-23 mice per group. Pups were generated from the breedings described in (A), arranged by maternal genotype. Data pooled from three independent experiments Statistical analysis was performed using a D'Agostino Pearson normality test to assess the normality of the samples, posteriorly using Student's t test or Mann-Whitney test. * - $p < 0,05$; ** - $p < 0,01$; *** - $p < 0, 001$; **** - $p < 0,0001$.

In pups from B cell-deficient mice, we observed a significant increase in the frequency of NK cells (Figure 3.9B) and a decrease in the frequency of alveolar macrophages and CD11b- DCs (Figure 3.9C). Alveolar macrophages and CD11b- DCs have been reported for having anti-inflammatory activities in the immune system (Shiokawa, Kotaki, Takano, Nakajima-Adachi, & Hachimura, 2017; Allard et al.,

2018). Accordingly, NK cells (Figure 3.9D) and neutrophils were increased in the absolute numbers in the pups born from B cell-deficient dams (Figure 3.9E). We found no differences in frequency and absolute numbers in T and B lymphocytes, and eosinophils.

Altogether, these results suggest that there is a bias for a type-1 immune response in pups born from B cell-deficient dams, evidenced by an increase of neutrophils and NK cells and cytokines such as IL-12p40 and IL12p70, while having a decrease in anti-inflammatory populations, such as alveolar macrophages and CD11b- DCs.

Our findings highlight the critical role of the maternal immune system in the offspring. The inability to produce antibodies or a deletion for a specific subset of immune cells, such as γδ T cells, not only affects the mothers, altering their microbiome, for example, but can dysregulate the offspring immune system. This dysregulation can affect the immune system of the offspring, through the increase in the production of certain cytokines and shaping the tissue resident immune cell populations, leading for a more type-1 or type-2 prone immune environment, ultimately influencing the response to invading pathogens.

4 – Discussion

4.1 – Discussion

In the present thesis we assessed the influence of the maternal immune system in the offspring, namely how the absence of cell populations responsible for mucosal immunosurveillance or antibody production impact on lung immunity. Here we showed that the absence of maternal γδ T cells leads to a type-2 biased immune environment in the lungs during steady state, with an increase in the type-2 cytokines IL-33 and IL-4, together with an increase in innate type-2 cytokine producers, IL-5+ MCs and IL-13+ ILC2s. Furthermore, upon N.b. infection, mice born from γδ T cell-deficient dams present an increase in immune cell infiltrate, composed mainly by type-2 immune cells, such as eosinophils, CD11b+ DCs and PD-L2-expressing DCs and macrophages. These alterations in the pulmonary milieu were abrogated upon antibiotic treatment, highlighting a role of the microbiota in the present model. However, a more detailed analysis of microbial communities across different tissues is needed in order to better understand the mechanisms behind this phenomenon. Importantly, the type-2 bias found in the lungs of the progeny of γδ T cell-deficient dams, are dependent solely on the maternal genotype, with no influences of the genotype of the offspring. Moreover, even though this phenotype seemed to be independent on antibody transfer, the total inability to produce immunoglobulins by the mothers, leads to an increase of type-1 cytokines of the IL-12 family, IL-12p40 and IL-12p70, in the lung of the offspring. Also, we found an increase in neutrophils and NK cells within pulmonary tissue, while cells with anti-inflammatory activities, such as alveolar macrophages and CD11b- DCs, were decreased. However, the mechanisms behind this phenotype and which class of immunoglobulins has a more relevant role in shaping offspring lung immunity is yet to be assessed.

Preliminary data from our group showed TCRδ$^{-/-}$ mice had increased pulmonary damage upon N.b. infection, when compared to wt mice. However, when using LMCs, no differences were found in pulmonary damage. The main difference between these two models, was the difference in the maternal genotype, the first being a pure knockout breeding (TCRδ$^{-/-}$ x TCRδ$^{-/-}$), while the latter was between γδ T cell-sufficient mice (TCRδ$^{+/-}$ x TCRδ$^{+/-}$). These results led us to hypothesize that the maternal genotype could influence the maturation of the offspring immune system, thus explaining this seemingly contradictory findings. The maternal influence in the immune system can be characterized in two main mechanisms, the transfer of maternal immunoglobulins during pregnancy and breastfeeding (Jennewein et al., 2017; Weaver et al., 1998), and through the transfer of microbiota (Zachariassen et al., 2018).

The use of LMCs ensure that the genetic background is normalized, with both groups of mice being genetically similar with the only exception for the targeted gene, and also, since they are born from the same mother, external confounding factors are normalized, since both groups are raised in the same environment (Holmdahl & Malissen, 2012). We have conducted a brief review of the literature regarding the use of LMC vs WT models in γδ T cells studies. Surprisingly, in the last 25 years, the majority of the studies using mouse models for γδ T cells were done using WT mice as controls, instead of LMCs (Figure 4.1). This might suggest that the majority of the findings for the last 25 years regarding γδ T cell functions did not take into account environmental factors or genetic drifting, possibly masking gene-extrinsic effects on the real functions of γδ T cells, which can generate some of the controversies in the literature about γδ T cells in lung pathologies, all due to the model used.

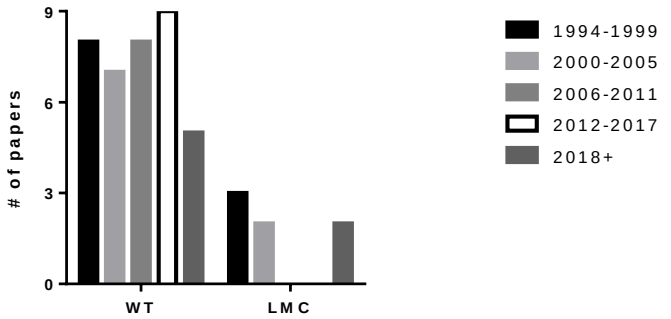

Figure 4.1 – Number of papers using WT or LMC mice models, for the last 25 years

During steady state, we observed an increase in type-2 cytokines and cells that produce type-2 cytokines, in pups born from γδ T cells-deficient dams. It has been reported by de Kleer and colleagues and also Saluzzo and colleagues, that in early life, IL-33 production increases in the lungs due to the mechanical stress produced by the first breath (Kleer et al., 2016; Saluzzo et al., 2017). Here we report, that the increase in IL-33 is also influenced by the maternal immune system through maternal transfer of microbiota. Interestingly, the increase of IL-33, leads to an increase in IL-13-producing and IL-5-producing MCs and ILC2s, which in turn polarize AM to a M2 phenotype, producing IL-4 (Saluzzo et al., 2017; Jang & Kim, 2015; Riedel et al., 2017). And, although we did not observe any differences in the numbers of AM in steady state, we did not assess their transcriptional programming. Furthermore, a decrease in the production of IL-27 was noticed in the lung of pups born from γδ T cells-deficient dams, this cytokine has been implicated in the inhibition of Th2 cells (Yoshimoto et al., 2007), thus the decrease of IL-27 expression in the lungs, further confirms the biased type-2 milieu in the lung of pups born from γδ T cell-deficient dams. Importantly, these changes, are dependent solely on the maternal genotype, since when comparing the pups according to their own genotype, no differences were observed, highlighting the importance of the maternal immune system in shaping the offspring immune environment.

Upon N.b. infection, we observed an increase type-2 immune cells, mainly eosinophils and PD-L2 expressing DCs and macrophages in pups born from γδ T cells-deficient dams. However, these differences were only found at day 6 p.i, while on day 2 p.i. no differences were found. Since the helminth N.b is known for inducing strong type-2 response (Voehringer et al., 2004), the increase in type-2 immune cells was expected. Although we did not assess cytokine production during N.b. infection, we can hypothesize, based on our steady state data, the type-2 cytokine expression is also increased in mice born from γδ T cells-deficient dams. A possible mechanism that could explain the increase in type-2 immune cells, can be due to the fact of N.b. infection inducing an increase in IL-33 expression (Hung et al., 2013), which promotes the migration of DCs to the lungs (Kleer et al., 2016). In turn, DCs are stimulated with N.b. secretions amplify the Th2 response (Ekkens et al., 2003) increasing the production of IL-4, stimulating DCs and macrophages to recruit eosinophils to the lungs (Voehringer et al., 2004), while stimulating PD-L2 expression in macrophages (Loke & Allison, 2003). Although this hypothesis needs to be assessed in the future.

According to our data, transfer of maternal microbiota is responsible for the differences we observed between the progeny from γδ T cell-deficient and -sufficient dams. The importance of the microbiota

composition has been related with the immune system. In a study by Chua and colleagues, it was observed in patients with respiratory allergies, an increased fecal abundance of *Ruminococcus gnavus*. To assess the potential role of *R. gnavus* in the pathogenesis of allergic diseases, the authors established an asthma mouse model, where they either sensitized mice with OVA, and injected *R. gnavus* or not, and assessed the airway response comparatively to a naïve mouse. The mice injected with *R. gnavus* and sensitized with OVA presented a more severe airway hyper-responsiveness and inflammation, accompanied by the increase in the expression of IL-33, ILC2s and eosinophils (Chua et al., 2018). This shows that one alteration in a species of bacteria that is present in the microbiome, is sufficient to an alteration of the immune system. The impact of maternal microbiota has been reported for inducing immune tolerance in pups, where a change in the birth method alters the microbiota of pups. In a study comparing different modes of delivery, it was shown that pups born from c-section, had an increase of *Prevotella* genus members and the *Ruminococcaceae* family in the gut microbiome, which lead to a decrease in activated Tregs in the MLN and the cytokine IL-10 levels (Zachariassen et al., 2018). Showing an impact of the maternal microbiota in the immune system of the offspring.

Another mechanism of maternal influence on the immune system, is the transfer of immunoglobulins. Immunoglobulins have different functions in the organism, although they share one main function, the protection of against pathogens. We report in pups born from B-cell deficient mothers, which do not produce immunoglobulins, an increase of type-1 cytokines, IL-12p40 and IL-12p70, and in immune populations, neutrophils and NK cells. IL-12 is a cytokine produced by DCs, monocytes, macrophages and B cells and has two main functions: generation of Th1 cells and inducing IFN-γ production, primarily by NK cells. In addition, IL-12 activates NK cells and cytotoxic T lymphocyte differentiation, while also acting as a direct chemotactic factor to NK cells infiltration, enhancing their numbers in the inflammatory site (Gee et al., 2009). Interestingly, there have been reports, that neutrophils produce IL-12 in a *Toxoplasma gondii* infection. When the authors depleted neutrophils in mice, they noticed a decrease in the serum levels of IL-12, when comparing these mice with the controls (Bliss, Butcher, & Denkers, 2000). We can hypothesize that the increase of neutrophils is increasing the production of IL-12, resulting in an increase of NK cells infiltration in the lungs of pups born from B cell-deficient dams.

Interestingly, we observed a decrease in immune population with reported anti-inflammatory activities, mainly CD11b- DCs and alveolar macrophages in the lung of pups born from B cell-deficient dams. There has been a report, of the properties of CD11b- DCs and their capacity to induce regulatory T cells (Tregs) in the gut. The authors propose the induction of Tregs is made through the production of TGF-β, since previous reports stated the capacity of DCs to produce this cytokine (Shiokawa et al., 2017). Also, there has been reports of the anti-inflammatory properties of alveolar macrophages. One function of alveolar macrophages is the capacity to phagocyte apoptotic cells, preventing the release of pro-inflammatory cytokines. In a study, when comparing AM-deficient mice, with -sufficient mice, the authors noticed an increase in inflammation, destruction of the lungs parenchyma, and the increase of the pro-inflammatory cytokines TNF-α and IL-1β (Allard et al., 2018; Knapp et al., 2003).

The relevance of maternal antibodies has been established for the maturation of the fetal immune system. Depending on the period in life, different immunoglobulins are passed to the offspring. IgG is mainly transported in the uterus to the fetus, while IgA in passed via milk during breast feeding (Jennewein et al., 2017; Weaver et al., 1998). One of the main functions of maternal immunoglobulins, mainly IgG, is the maturation of B cells. It has been reported, on pups born from IgG-deficient dams presented a decrease in of B and pre-B cells in the bone marrow, ultimately leading to a decrease in the immunoglobulin levels (Malanchère et al., 1997). On the other hand, maternal IgG and IgA have been reported to dampen T-cell dependent immune responses against commensal bacteria, preventing the

polarization of T naïve cells to T effector, that promote B cell differentiation, secreting immunoglobulins against the gut microbiota (Koch et al., 2016). However, we had no differences in B cells and T cells; although we only looked in the lungs. Furthermore, neutrophils can bind to different classes of immunoglobulins, mainly IgG and IgA, this is due to these cells expressing FcγRII and FcγRIIIB, being capable of opsonizing bacteria, in a *Staphylococcus aureus* infection model (Kok et al., 2014). Also, NK cells express FcγRIII, which is needed for their antibody-dependent cellular cytotoxicity (ADCC) activity. In a γ-deficient mice model, the loss of of FcγRIII was shown to abrogate almost completely ADCC activity in NK cells (Takai, Li, Sylvestre, Clynes, & Ravetch, 1994). These findings, in addition to our results, suggest that even though neutrophils and NK cells are increased in the lungs of pups born from B cell-deficient dams, they are poorly activated, although further studies are needed to confirm this hypothesis.

4.2 – Implications

The work presented in this thesis, highlights the relevance of the maternal immune system in newborns. We report the importance of LMCs in scientific research, in contrast to WT models. Having the objective of assessing the impact of one gene/cell type in mouse models, the genetic background and possible environmental influences cannot be ignored. Breeding LMCs is a cleaner model, since, the differences caused by our target gene, are not diluted by potentials genomic alterations, that arise from several generations of crossing for one genotype, and by external influences, that came from being inserted in different environments, producing phenotypes that might not correspond to the truth. However, the use of LMCs are not common practice in scientific research in the role of γδ T cells in infection, taking the example of the studies on γδ T cells for the past 25 years, having the vast majority used WT mouse models.

Although being shown for the gut, by Gomez de Agüero and colleagues, the role of the mother in shaping the immune system of the offspring (Agüero et al., 2016), this has never been shown before to the lungs. Furthermore, the type-2 biased lungs environment of pups was thought to be caused by the mechanical stress (Saluzzo et al., 2017). Here we show that, the maternal immune system has a role in the pulmonary environment of neonates, contributing for the type-2 biased milieu. Furthermore, depending on the maternal immune system, the response to a pathogen, such as N.b. is altered. This shows the importance of the maternal immune system in the education of the offspring immune system, mainly in the capacity to respond to external stimuli. Our results shed new light on the importance of the maternal immune system in neonates, influencing their immune environment.

The balance existing between the immune system and microbiota has been implicated in several studies, where an alteration in a single bacterium in the microbiome, can lead to a destructive effect, causing different pathologies (Trompette et al., 2014; Nyangahu & Jaspan, 2019). The modulation of our microbiome can be done through our habits, for example the consumption of antibiotics, that not only kill pathological bacteria, but also affect our commensal bacteria. Our microbiome, as stated previously, can educate our immune system, due to several, direct or indirect, interactions. Our study sheds new light on some alterations the lack of γδ T cells can induce some alterations in the maternal microbiota, thus leading to a more type-2 biased pulmonary immune environment. These results might suggest, when using mice model, an extra care may be needed to not alter the mice microbiota, which can lead to some results being influenced by these changes, and not by the variables that we are assessing.

Maternal immunoglobulins are transferred to the offspring during pregnancy and breastfeeding. The lack of maternal immunoglobulins, as we presented in this thesis, leads to some differences in the

immune system, specifically a type-1 biased pulmonary immune environment, and a decrease in anti-inflammatory immune populations. This information is relevant for breastfeeding, providing an argument that supports privileging breastfeeding against formula powder during early life, providing a balance in the immune system, with a normal development. Also, these findings strengthen the importance in the maternal immune system in modulating the offspring immune system.

4.3 – Future work

To further elucidate the impact of the maternal microbiota and maternal immunoglobulins, some aspects need to be assessed in the future.

Having a difference in cytokine milieu, at steady state it would be interesting to assess the T cell differentiation and the cytokine profile during infection, since we report alterations in the cytokine profile during steady state of pups born from TCR$\delta^{-/-}$ dams, mainly in cytokines that are known to be produced by Th2 and to further characterize the pulmonary immune system (Kips, 2001).

For the maternal microbiota, it would be interesting to assess which modifications occurred in the microbiota, if we can identify which bacteria are different or if is an alteration of the diversity of the microbiota that is affected. Furthermore, it would be useful to understand which specific bacteria are responsible for the changes in the pups. For this we would analyze the microbiota of different organs in the mice, skin or fecal, for example, and if the birth method, natural birth vs c-sec, has an impact, in the pulmonary immune environment. To further validate our results, a system using germ-free mice, would help understanding the impact of maternal γδ T cell, without the microbiota having an impact.

Also, it would be important to assess if microbial products are different among pups from γδ T cell-deficient and γδ T cell-sufficient dams, in order to understand how the microbiota is modulating the immune system of the pups.

For the maternal immunoglobulins, it would be interesting to study if the causes of the impact are caused by the immunoglobulins transferred in uterus or through breastmilk. This could be done by doing a cross-fostering, where we would exchange the pups, from the JHT$^{-/-}$ dams with the WT dams, and *vice-versa*.

We would like to assess the immune system of the pups, upon infection. Since there is a type-1 biased pulmonary immune environment, a viral infection, such as Influenza infection would be interesting to assess the immune response to a pathogen, having differences in steady state. Also, in both steady-state and during infection, it would be useful to assess the polarization of T cells and the state of maturity of B-cell in the pups, since we have differences in IL-12 which polarize Th0 into Th1 (Gee et al., 2009), and maternal immunoglobulins are known to affect B cell development (Malanchère et al., 1997).

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7 – Supplementary figures

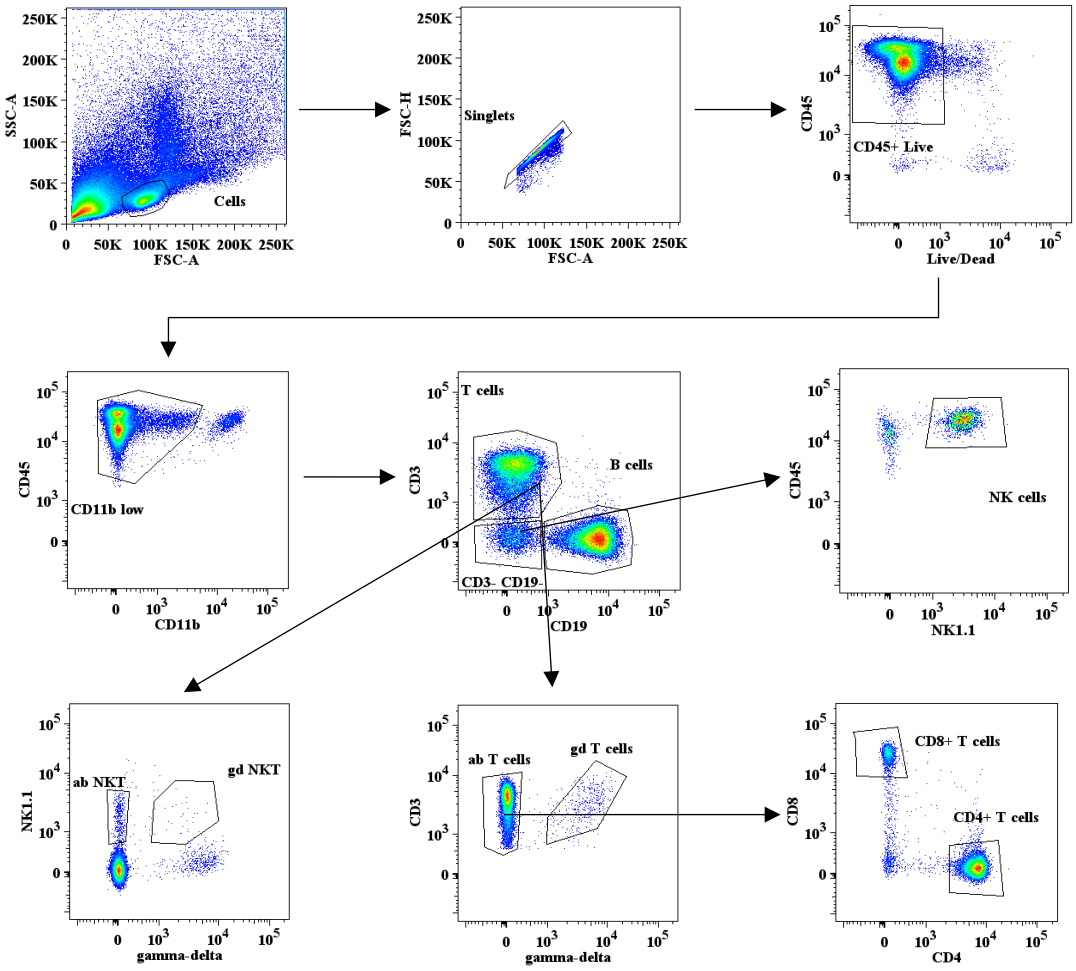

Figure 7.1 – Gating strategy for lymphoid cells

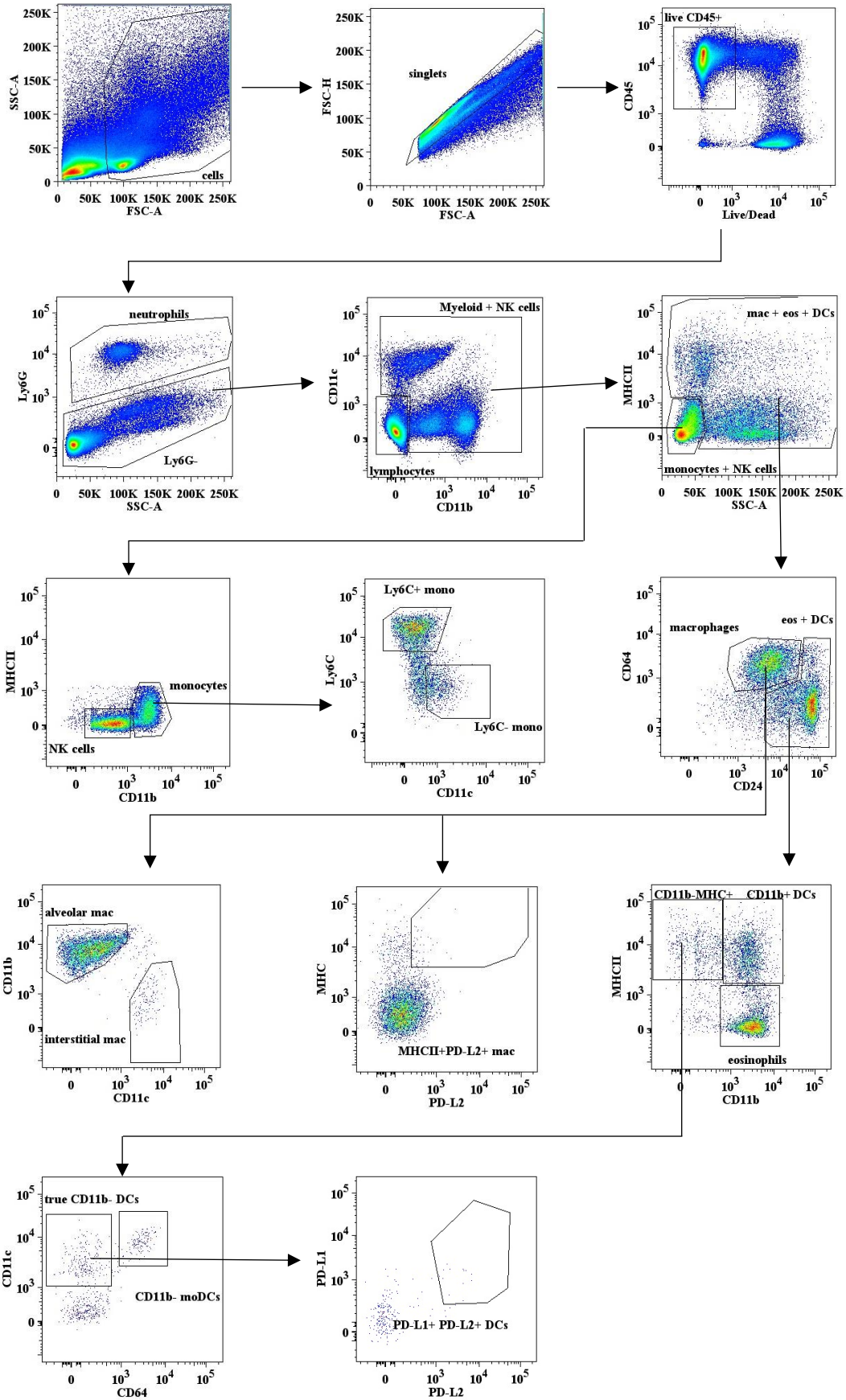

Figure 7.2 – Gating strategy for Myeloid cells

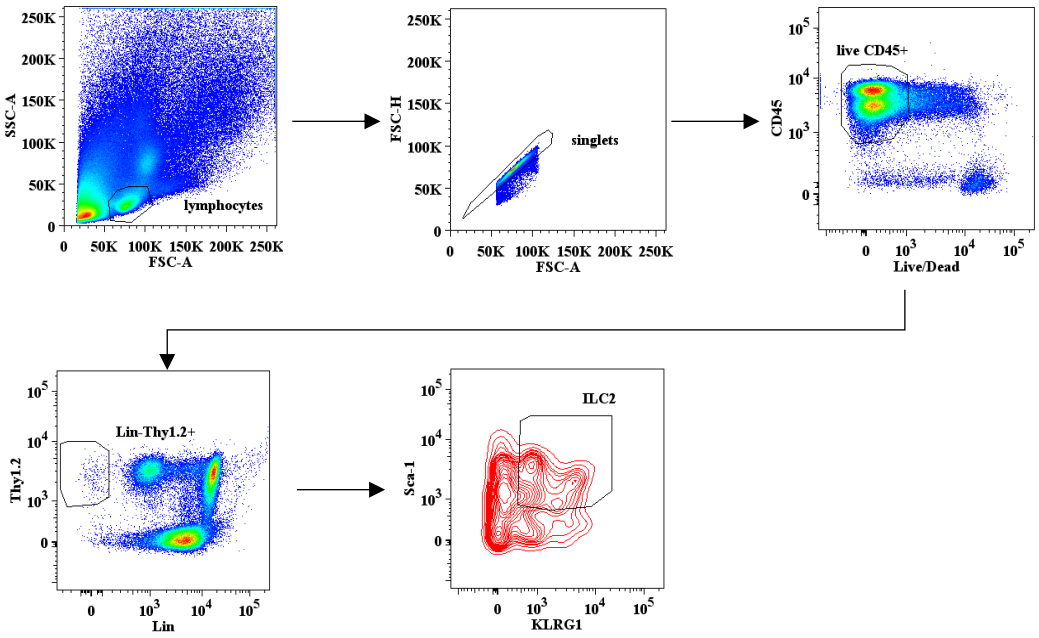

Figure 7.3 – Gating strategy for ILC2s

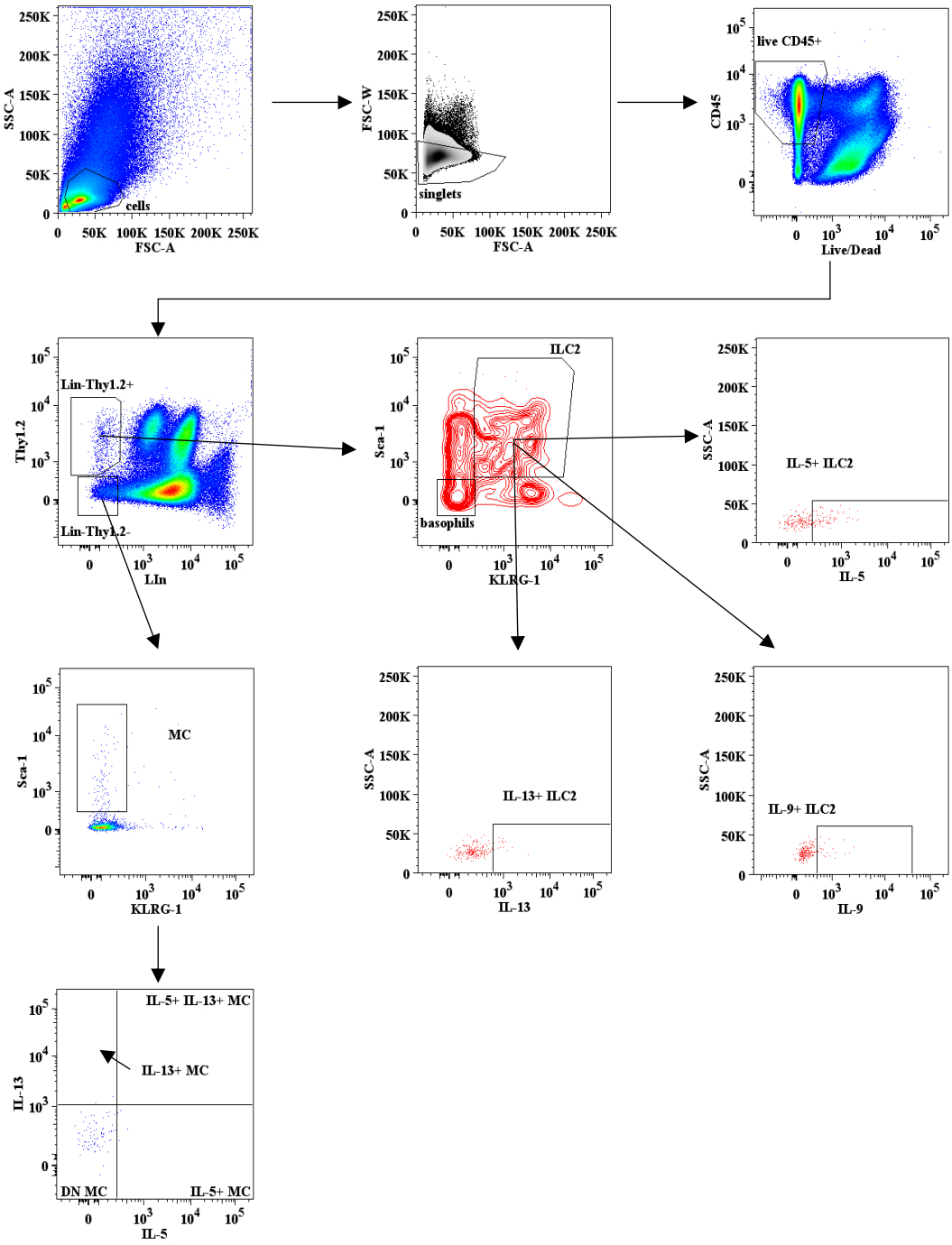

Figure 7.4 – Gating strategy for IL-13, IL-9 and IL-5 producing cells